"Immunoglobulin Fusion Proteins"

Field of the Invention

This invention relates generally to methods for constructing chimeric proteins and more specifically to methods for constructing recombinant immunoglobulin fusion proteins.

Background of the Invention

Prolonging the circulating half-lives of protein pharmaceuticals is of interest to patients and healthcare providers. Long acting protein therapeutics should require less frequent injections and can be effective at lower doses than proteins with shorter circulating half-lives. It is known that increasing the effective size of a protein can increase its circulating half-life by preventing removal of the protein by the kidney (Knauf et al., 1988; Mahmood, 1998). One method that can be used to increase the effective size of a protein is to use recombinant DNA technology to covalently fuse the protein of interest to a second protein. The larger fusion protein often has a longer circulating half-life than the non-fused protein (Capon et al., 1989; Zeng et al., 1995). One class of proteins that has been used frequently to create fusion proteins is immunoglobulins (Ig), which are major components of blood. Immunoglobulins occur in various classes known as IgG, IgM, IgA, IgD, and IgE (Roitt et al., 1989). Human IgGs can be further divided into various types known as IgG1, IgG2, IgG3 and IgG4, which are products of distinct genes. IgG1 is the most common immunoglobulin in serum (70% of total IgG) and has a serum half-life of 21 days (Capon et al., 1989; Roitt et al., 1989). Although less abundant, IgG4 also has a long circulating half-life of 21 days (Roitt et al., 1989)

Human IgGs have a multidomain structure, comprising two light chains disulfide-bonded to two heavy chains (referred to herein as a "tetramer"; reviewed in Roitt et al., 1989). Each light chain and each heavy chain contains a variable region joined to a constant region. The variable regions are located at the N-terminal ends of the light and heavy chains. The heavy chain constant region is further divided into CH1, Hinge, CH2 and CH3 domains. The CH1, CH2 and CH3 domains are discreet domains that fold into a characteristic structure. The Hinge region is a region of considerable flexibility. Flexibility of the hinge can vary depending upon the IgG isotype (Oi et al., 1984; Dangl et al., 1988). IgG heavy chains normally form disulfide-linked dimers through cysteine residues located in the Hinge region. The various heavy chain domains are encoded by different exons in the IgG genes (Ellison et al., 1981; 1982).

Proteins have been fused to the heavy chain constant region of IgGs at the junction of the variable and constant regions (thus containing the CH1-Hinge-CH2-CH3 domains - referred to herein as the IgG-C_H fusions) at the junction of the CH1 and Hinge domains (thus containing the Hinge-CH2-CH3 domains - referred to herein as IgG-Fc fusions), and at the C-terminus of the IgG heavy chain (referred to herein as IgG-C-terminal fusions).

IgG fusion proteins have been created most often with the extracellular domains of cell surface receptors (reviewed in Chamow and Ashkenaki, 1996). Examples of extracellular domains of cell surface receptors that have been joined using recombinant DNA technology to the C_H or Fc domains of human or mouse IgGs include CD4 (Capon et al., 1989), tumor necrosis factor receptors

(Mohler et al., 1993), CTLA4 (Linsley et al., 1991a), CD80 (Linsley et al., 1991b), and CD86 (Morton et al., 1996). Extracellular domains of receptors evolved to function when fused to other amino acids, i.e., the transmembrane and intracellular domains of the receptor; therefore it is not surprising that extracellular domains retain their ligand binding properties when fused to other protein domains such

5   as IgG domains. Despite this, differences in ligand binding properties have been noted for certain extracellular domains. For example, a fusion protein comprised of the extracellular domain of CD4 to human IgG1-C_H had 2-fold reduced affinity for the CD4 ligand gp120 than non-fused CD4 (Capon et al., 1989).

There are far fewer examples of proteins that are normally soluble, e.g., growth factors,

10   cytokines and the like, which have been fused to IgG domains and retained full biological activity. Soluble proteins did not evolve to function when fused to other proteins and there is no reason to expect them to retain biological activity when fused to other proteins. In fact, in the majority of the published examples, biological activity of the fused cytokine/growth factor was significantly reduced relative to the non-fused cytokine/growth factor. Whether or not the cytokine/growth factor will

15   function properly when fused to another protein will depend upon many factors, including whether the amino-terminus or carboxy-terminus of the cytokine/growth factor is exposed on the surface of the protein, whether these regions are important for biological activity of the cytokine/growth factor and whether the cytokine/growth factor is able to fold properly when fused to another protein. By their very nature, such factors will be highly protein-specific and unpredictable. Results with the few growth

20   factor/cytokine fusion proteins that have been studied have shown how protein-specific biological activity of the fusion protein can be. In the majority of cases, biological activity of the fused growth factor/cytokine is severely reduced, whereas, in the minority of cases full biological activity of the growth factor/cytokine is retained. In one case where biological activity of the fusion protein was significantly reduced, modifying the amino acids at the junction between the cytokine/growth factor

25   and the IgG domain resulted in a fusion protein with improved biological activity (Chen et al., 1994) This same modification did not improve biological activity of a second cytokine fused to the same IgG domain. [(Chen et al., 1994)

Growth factors that have been fused to IgGs include keratinocyte growth factor (KGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF-I). A KGF-mouse IgG1-Fc fusion

30   protein was created by LaRochelle et al. (1995). On a molar basis, the fusion protein was 4-5-fold less active than KGF in stimulating proliferation of Balb/MK cells in an *in vitro* bioassay. The KGF-IgG-Fc fusion also had approximately 10-fold lower affinity for the KGF receptor on cells than did KGF. A fibroblast growth factor-human IgG-Fc fusion was constructed by Dikov et al. (1998). On a molar basis the FGF- IgG1-Fc fusion protein was approximately 3-fold less active than FGF in *in vitro* assays

35   in stimulating DNA synthesis in NIH 3T3 cells. Shin and Morrison (1990) fused IGF-I to the C-terminus of IgG and found that the IGF-I-IgG C-terminal fusion protein had less than 1% of the *in vitro* bi logical activity f IGF-I.

Examples of cytokines that have been fused to IgG domains include IL-2, IL-4, IL-10 and GM-CSF. Landolphi (1991; 1994) described an IL-2-IgG1-C_H fusion protein, which included an extra

serine between the C-terminus of IL-2 and the N-terminus of the IgG-C_H domain. The IL-2-IgG1-C_H fusion protein was purported to be as active, on a molar basis, in *in vitro* bioassays as IL-2, but no details were provided as to how protein concentrations were quantitated (Landolphi, 1991; 1994). Zeng et al. (1995) fused mouse IL-10 directly to the Fc region of mouse IgG2a; however the first amino acid of the Fc hinge region was changed from Glu to Asp. Zeng et al (1995) reported that the IL-10-IgG2a fusion protein was fully active in *in vitro* bioassays; however, only two concentrations of the fusion protein were studied, both of which were saturating. Given these high protein concentrations, only major differences (e.g., 100-fold) in bioactivities between the IL-10-mouse IgG2a fusion protein and IL-10 could have been detected. To detect smaller differences in bioactivities, one needs to analyze serial dilutions of the proteins in *in vitro* bioassays and calculate $EC_{50}s$ (the concentration of protein required for half-maximal stimulation) or $IC_{50}s$ (the concentration of protein required for half-maximal inhibition). $EC_{50}s$ of the IL-10-IgG2a and IL-10 were not reported by Zheng et al. (1995). Chen et al.(1994) also constructed an IL-2-IgG fusion protein and reported that this fusion protein was fully active. Gillies et al. (1993) also reported creating a fully active IL-2 fusion protein comprising IL-2 fused to the C-terminus of an antiganglioside IgG antibody. Unexpectedly, Gillies et al. (1993) found that a fusion between the same antibody and GM-CSF displayed only 20% of wild type GM-CSF bioactivity. Chen et al. (1994) were able to create a fully active IgG-C-terminal-GM-CSF fusion protein by inserting four amino acids between the antibody molecule and GM-CSF. Unexpectedly, they reported that fusion of IL-4 to the same antibody using the same four amino acid linker resulted in an IL-4 protein with 25-fold reduced biological activity (Chen et al., 1994).

Qiu et al. (1998) described homodimeric erythropoietin (EPO) proteins in which two EPO proteins were fused together using flexible peptide linkers of 3-7 glycine residues. The peptide linker joined the C-terminus of one EPO protein to the N-terminus of the second EPO protein. In vitro bioactivities of the fusion proteins were significantly reduced (at least 4- to 10-fold) relative to wild type EPO (Qiu et al., 1998).

Thus, the literature indicates that the amino acids at the junction between the growth factor/cytokine domain and the IgG domain can have a profound influence on the biological activity of the fused growth factor/cytokine.

For use as human therapeutics, it is desirable that bioactivity of a growth factor/cytokine-IgG fusion protein be as close to wild type, i.e., the non-fused protein, as possible. Fusion proteins with high activity can produce greater therapeutic benefits at lower doses than fusion proteins with lower specific activities. This will reduce the cost of medicines and provide patients with greater therapeutic benefits. For use as a human therapeutic it also is advantageous that the protein be as homogeneous and pure as possible. Ig fusion proteins are synthesized as monomers, which can assemble to form disulfide-linked dimers and tetramers, depending upon the type of fusion protein. The relative proportions of monomers, dimers and tetramers can vary from lot to lot, depending upon manufacturing conditions. Lot to lot variability can result in heterogeneous product mixtures with varying activities. Because of their larger size, tetramers are expected to have longer circulating half-lives in the body than dimers r monomers, and dimers are expected to have longer circulating half-

lives than monomers. For this reason, tetramers, dimers and m nomers may produce different therapeutic effects *in vivo*. Purified, homogeneous preparati ns of tetramers, dimers or monomers are preferred as therapeutics because they will have defined specific activities. For use as human therapeutics it also may be beneficial to keep the length of any linker sequence joining the growth

5   factor/cytokine domain to the Ig domain to a minimum to reduce the possibility of developing an immune response to the non-natural amino acids in the linker sequence. Joining the two proteins without an intervening linker sequence, referred to herein as a "direct fusion", eliminates the possibility of developing an immune response to a non-natural linker and thus is one preferred form of an Ig fusion protein.

10   The literature on Ig fusion proteins merely provides examples for making "non-direct" cytokine/growth factor-Ig fusion protein, i.e., fusion proteins in which the growth factor/cytokine is joined to an Ig domain through a peptide linker. WO 99/02709 provides examples for joining EPO to an Ig domain by using a *Bam* HI restriction enzyme site to join DNA sequences encoding the proteins. This method can be used to create EPO-Ig fusion proteins containing linkers. Because *Bam* HI has a

15   specific DNA recognition sequence, GGATCC, which is not present at the junctions of the EPO or Ig-Fc, Ig-C_H or light chain constant regions, this method is not useful for constructing EPO-Ig direct fusions.

EP 0464533A provides examples for making Ig fusion proteins that contain the specific three amino acid linker, AspProGlu, between the cytokine and Ig domains. The methods described in EP

20   0464533A rely on RNA splicing of separate exons encoding the cytokine and the Ig domain to create the fusion protein. The AspProGlu linker was required at the end of the cytokine/growth factor coding region because the RNA splicing reaction has specific sequence requirements at the splice site; the AspProGlu linker is encoded by nucleotides that may allow RNA splicing to occur. EP 0464533A provides an example for making an EPO-Ig fusion protein containing an AspProGlu linker using this

25   method. The EPO coding region was modified to delete Arg166 and add the amino acid linker, ProGlu, immediately following Asp165 of the EPO coding sequence. This resulted in the creation of an EPO-Ig fusion protein containing an AspProGlu linker (Asp165 of the EPO coding sequence provided the Asp of the linker sequence). The methods described by EP 0464533A are, therefore, not useful for creating Ig direct fusions or Ig fusions that do not contain AspProGlu or ProGlu linkers.

30   US Patent No. 5,349,053 describes methods for creating an IL-2-IgG-C_H fusion protein that contains a Ser linker between the IL-2 and Ig domains. The methods described in US Patent No. 5,349,053 rely on RNA splicing of separate exons encoding the IL-2 and the IgG-C_H domain. Because the RNA splicing reaction has specific sequence requirements at the splice site, the IL-2 coding region needed to be modified to add a TCA codon encoding serine to the carboxy terminus of the IL-2 coding

35   region. The TCA codon was engineered to be followed by an intron splice junction sequence. This resulted in the creation of an IL-2-IgG-C_H fusion protein containing a Ser linker. The methods described in US Patent No. 5,349,053 are n t useful for creating cytokine/growth factor-Ig direct fusions, or cytokine/growth factor-Ig fusions that do not contain a Ser linker.

Growth factor/cytokine-IgG fusion proteins, including EPO-IgG and G-CSF-IgG fusion proteins, are contemplated in EP 0 464 533 A, WO99/02709 and Landolphi (1991; 1994). None of these references present any data regarding expression, purification and bioactivities of the contemplated EPO-IgG and G-CSF-IgG fusion proteins. Landolphi (1991; 1994) present bioactivity

5   data for an IL-2-IgG1-C_H fusion protein, but not for any other IgG fusion protein. The EPO-IgG1-Fc fusion protein contemplated by EP 0 464 533 A contains the two amino acid linker, ProGlu, between Asp165 of the EPO coding sequence and the beginning of the IgG1-Fc hinge region. The carboxy-terminal amino acid in EPO, Arg166 would be deleted. EP 0 464 533 A does not provide any information as to how the G-CSF coding region would be joined to the IgG1-Fc hinge region.

10   Bioactivity data for neither protein is provided in EP 0 464 533. WO 99/02709 describes an EPO-mouse IgG2a-Fc fusion protein, but not an EPO-human IgG fusion protein. Bioactivity data for the EPO-mouse IgG2a-Fc fusion protein are not presented. WO 99/02709 also does not provide details as to the source of the EPO cDNA or human IgG genes used to construct the contemplated fusion proteins or the precise amino acids used to join EPO to the IgG domain. The reference cited in WO 99/02709

15   (Steurer et al., 1995) also does not provide this information.

WO 99/02709 postulates that a flexible peptide linker of 1-20 amino acids can be used to join EPO to an Ig domain; however the amino acids to be used to create the flexible linkers are not specified in WO 99/02709. Qiu et al. (1998) reported that EPO fusion proteins joined by flexible linkers of 3-7 glycine residues have significantly reduced biological activities (4-10-fold) relative to wild type EPO.

20   WO 99/02709 provides methods for using conditioned media from COS cells transfected with plasmids encoding hypothetical EPO-IgG fusion proteins to increase the hematocrit of a mouse. Such conditioned media will contain mixtures of monomeric and dimeric EPO-IgG fusion proteins as well as many other proteins. WO 99/02709 does not provide any evidence demonstrating that the methods taught are effective.

25   Thus, despite considerable effort, a need still exists for improved methods for creatinggrowth factor/cytokine-Ig fusion proteins directly or with a variety of different linker types that preserve biological activity of the growth factor/cytokine domain of the Ig fusion protein. The present invention satisfies this need and provides related advantages.

30   Brief Description of the Drawing

Figure 1 is a diagram showing the amplification of two DNA fragments (CH1-Hinge and Hinge-CH2-CH3) that share a region of partial overlap (a portion of the Hinge region) using PCR and oligonucleotide primers a and b to generate a larger DNA fragment that includes CH1-Hinge-CH2-CH3.

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Summary of the Invention

The present invention discloses novel Ig fusion proteins in which a soluble protein is j ined directly to an Ig domain or by an amino acid linker that is not AspProGlu or Ser. The soluble protein is a growth factor, a cytokine r an active variant f a growth factor r cytokine. Useful linkers include a

mixture of Glycine and Serine residues, preferably SerGly and Ser(GlyGlySer)$_n$(SEQ.ID.NO.1), where n can be 1-7. The Ig domain preferably includes the constant region and is preferably selected from IgG-Fc, IgG-C$_H$ or C$_L$. Examples of growth factors and cytokines and their active fragments that substantially retain biological activity of the non-fused proteins when joined to Ig domains as direct fusions or through these linker sequences include members of the growth hormone (GH) supergene family such as, for example, G-CSF, EPO, GH, alpha interferon, beta interferon, gamma interferon, GM-CSF, IL-11, TPO, SCF and Flt3 ligand.

For example, EPO-IgG-Fc fusion proteins and G-CSF-IgG-Fc fusion proteins have biological activities, on a molar basis, equivalent to non-fused EPO and G-CSF. IgG-C$_H$ fusions of EPO and G-CSF surprisingly have in vitro biological activities within 2-4-fold of non-fused EPO and G-CSF. Growth Hormone-IgG-Fc and Growth Hormone-IgG-C$_H$ fusion proteins unexpectedly have biological activities within 4- to 17-fold of non-fused Growth Hormone. Similarly, biologically active IgG-Fc, IgG-C$_H$, and Ig-C$_L$ fusion constructs using IFN-α2 and IFN-β are also provided.

IgG-Fc fusions of EPO, G-CSF and Growth Hormone are more active than identical IgG-C$_H$ fusions of these growth factors/cytokines. Ig-C$_L$ fusions of IFN-α are more active than identical IgG-Fc or IgG-C$_H$ fusions of IFN-α. The bioactivity differences between IgG-Fc, IgG-C$_H$ and Ig-C$_L$ fusion proteins were also unexpected. In addition, preferred sites for constructing EPO-IgG, G-CSF-IgG, Growth Hormone-IgG, and IFN-α-Ig fusion proteins that maximize bioactivity of the fused EPO, G-CSF, Growth Hormone and IFN-α domains have now been identified.

The invention further provides novel growth factor/cytokine-Ig fusion proteins comprising multimers of heavy and light chain fusion proteins as well as methods for preparing preparations of dimeric growth factor/cytokine-Ig fusion proteins that are essentially free of monomeric fusion proteins, aggregates and contaminating proteins. "Essentially free" is used herein to mean at least 90% pure. The invention also provides novel multimeric growth factors/cytokines where two or more cytokines/growth factors are joined as direct fusions or by using an amino acid linker.

Methods of treating various conditions are also provided in which an effective amount of an Ig fusion protein of the present invention are administered to a patient in need of such therapy.

Detailed Description of the Invention

In work described herein, it has now been discovered that bioactivities of growth factor/cytokine-Ig fusion proteins can vary depending upon the isotype of the IgG domain, where the growth factor/cytokine is attached to the IgG domain, and whether the protein is attached to the constant region of an Ig light chain or an Ig heavy chain. These results were not predicted from the literature. Although not wishing to be bound by any particular theory, the inventors postulate that the bioactivity variability they observed may relate to the flexibility and other physical properties of the region in the Ig domain used to join to the fusion proteins. Based upon these findings, it is believed that bioactivity of an Ig fusi n proteins created using mouse Ig domains cannot be used to predict bioactivity of a Ig fusion proteins created human Ig d mains.

Accordingly, the invention generally relates to novel methods for constructing Ig fusion proteins comprising a soluble protein joined to an Ig domain and to the novel Ig fusion proteins btained by the methods. The methods involve the joining of a soluble protein to an Ig domain as a direct fusion or with an intervening amino acid linker.

5   The methods of the present invention are useful for constructing Ig fusion proteins containing one or more growth factors and/or cytokines, including, without limitation, EPO, Growth Hormone (GH), G-CSF, IL-11, thrombopoietin (TPO), Stem Cell Factor, Flt3 Ligand, GM-CSF and interferon, especially alpha, beta and gamma interferon. Other proteins for which the methods are useful include other members of the GH supergene family. Members of the GH supergene family include GH,
10   prolactin, placental lactogen, erythropoietin (EPO), thrombopoietin (TPO), interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 (p35 subunit), IL-13, IL-15, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, alpha interferon, beta interferon, gamma interferon, omega interferon, tau interferon, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF),
15   cardiotrophin-1 (CT-1), Stem Cell Factor and the flt3/flk2 ligand (Bazan (1990; 1991; 1992; Mott and Campbell (1995); Silvennoinen and Ihle (1996); Martin et al., 1990; Hannum et al., 1994). It is anticipated that additional members of this gene family will be identified in the future through gene cloning and sequencing. Members of the GH supergene family have similar secondary and tertiary structures, despite the fact that they generally have limited amino acid or DNA sequence identity. The
20   shared structural features of members of the GH supergene family, which are described in Bazan (1990; 1991; 1992), Mott and Campbell (1995) and Silvennoinen and Ihle (1996), allow new members of the gene family to be readily identified. The methods described herein also are useful for creating Ig fusion proteins with proteins that are normally soluble and are not members of the GH supergene family. Active variants of these proteins can also be used in constructing the Ig fusion proteins of the
25   present invention. The term "active variant" means a fragment of a soluble protein that substantially retains the biological activity of the full length protein and is described in more detail below.

The methods disclosed herein also can be used to join a growth factor/cytokine to an Ig domain using a linker of any amino acid sequence and of any length as long as the linkers are not AspProGlu or Ser. Preferred linkers are 1-50 amino acids in length, and more preferably 1-22 amino
30   acids in length. Preferably the linker will not adversely affect bioactivity of the growth factor/cytokine or Ig domain. Preferred linkers contain mixtures of glycine and serine residues. Other amino acids that could be incorporated into useful linkers include threonine and alanine residues. One preferred linker disclosed herein is the two amino acid linker SerGly. Other preferred linkers disclosed herein are linkers of the motif Ser(GlyGlySer)$_n$(SEQ.ID.NO.1) where n can be 1-7.

35   The Ig domains of the present invention include the constant region such as, for example, an IgG-Fc, IgG-C$_H$, an Fc or C$_H$ domain from another Ig class,i.e., IgM, IgA, IgE, IgD or a light chain constant domain. Truncations and amin acid variants or substitutions of these domains also are included in this invention.

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In another aspect f the invention, IgG-Fc and IgG-C$_H$ fusion proteins, for example, are synthesized as monomers that can assemble to form dimers. Typically, the dimers are joined by disulfide bonds in the IgG Hinge region. Conditioned media from cells secreting the IgG fusion proteins can contain mixtures of IgG fusion protein monomers and dimers. For use as human

5   therapeutics it will be desirable to use homogeneous populations of either IgG fusion protein monomers or dimers, but not mixtures of the two forms. Methods for obtaining essentially pure preparations of dimeric growth factor/cytokine-IgG fusion proteins are also provided. The methods are generally accomplished by obtaining a host cell capable of expressing the IgG fusion protein, collecting the conditioned media, and purifying the dimeric fusion protein from monomeric fusion protein,

10   aggregates and contaminating proteins by column chromatography procedures. Suitable host cells for expressing the IgG fusion proteins include yeast, insect, mammalian or other eukaryotic cells. Preferably, the host cell is a mammalian cell, particularly COS or CHO cells.

In one embodiment, EPO is joined directly to an Ig-Fc domain, for example an IgG-Fc domain, and has an EC$_{50}$ less than 1,000 ng/ml, preferably less than 100 ng/ml, more preferably less

15   than 10 ng/ml and most preferably less than 4 ng/ml. In another embodiment EPO is preferably joined to an IgG-Fc or IgG-C$_H$ domain through a peptide linker of 2 to 7 amino acids and has an EC$_{50}$ less than 1,000 ng/ml, preferably less than 100 ng/ml, more preferably less than 10 ng/ml and most preferably less than 4 ng/ml.

In another embodiment, G-CSF is joined directly to an Ig-Fc domain, for example an IgG-Fc

20   domain, and has an EC$_{50}$ less than 300 ng/ml, preferably less than 30 ng/ml, more preferably less than 3 ng/ml and most preferably less than 300 pg/ml. In another embodiment G-CSF is preferably joined to an IgG-Fc or IgG-C$_H$ domain through a peptide linker of 2 to 7 amino acids and has an EC$_{50}$ less than 300 ng/ml, preferably less than 30 ng/ml, more preferably less than 3 ng/ml and most preferably less than 300 pg/ml.

25   In another embodiment, GH is joined to an Ig-Fc or Ig-C$_H$ domain, for example an IgG-Fc or IgG-C$_H$ domain, through a peptide linker of 2 to 7 amino acids and has an EC$_{50}$ less than 1,000 ng/ml, preferably less than 100 ng/ml, and most preferably less than 10 ng/ml.

In another embodiment, IFN-α is joined to an Ig-Fc or Ig-C$_H$ domain, for example an IgG-Fc or IgG-C$_H$ domain, through a peptide linker of 2 to 7 amino acids and has an IC$_{50}$ less than 1,000

30   ng/ml, preferably less than 100 ng/ml, more preferably less than 40 ng/ml and most preferably less than 10 ng/ml. In another embodiment IFN-α is joined to a light chain constant region though a peptide linker of 2 to 7 amino acids and the fusion protein has an IC$_{50}$ less than 100 ng/ml, preferably less than 10 ng/ml, more preferably less than 1 ng/ml and most preferably less than 100 pg/ml.

In another embodiment, IFN-β is joined to an IgG-Fc or IgG-C$_H$ domain through a peptide

35   linker of 2 to 7 amino acids and has an IC$_{50}$ less than 10,000 ng/ml, preferably less than 1,000 ng/ml, more preferably less than 100 ng/ml and most preferably less than 30 ng/ml.

It has now been discovered that purified GH-, EPO- and G-CSF-IgG-C$_H$ fusion proteins have reduced specific activities in *in vitro* biol gical assays compared to th non-fused proteins and IgG-Fc fusion proteins. The invention further provides novel, multimeric IgG-C$_H$ /Ig light chain constant

domain fusion proteins with improved biol gical activities. The invention also relates to growth factor/cytokine-IgG fusion proteins in which the IgG domain has been altered to reduce its ability to activate complement and bind Fc receptors. The alteration of the IgG domain for this purpose is described in Example 6 below.

5   Novel multimeric growth factor and/or cytokine fusion proteins are also provided. In one embodiment two or more growth factors and/or cytokines are joined directly to each other without an intervening peptide linker. In another embodiment two or more growth factors and/or cytokines are joined to each other through a peptide linker of 1 to 50 amino acids, and more preferably through a peptide linker of 1 to 7 amino acids. Particularly useful linkers for this purpose include SerGly and

10   Ser(GlyGlySer)$_n$(SEQ.ID.NO.1), where n can be 1-7.

        This invention includes active variants of the proteins and immunoglobulin domains that possess some or all of the biological properties of the corresponding full length or wild-type proteins or immunoglobulin domains. Such variants include, but are not limited to, amino acid variants, amino acid substitutions, truncations, additions, changes in carbohydrate, phosphorylation or other attached

15   groups found on natural proteins, and any other variants disclosed herein. In some cases the variants may possess additional properties, e.g., improved stability or bioactivity, not shared by the original protein. For example, Mark et al. (1984) disclose an IFN-β mutein in which cysteine at position 17 is replaced by serine. IFN-β (Ser-17) displays improved stability relative to wild type IFN-β. Similarly, replacing cysteine-125 of IL-2 yields a more stable IL-2 protein (Landolphi, 1991; 1994). Lu et al.

20   (1992) disclose a G-CSF mutein in which cysteine at position 17 is replaced by serine. Kuga et al. (1989), Hanazono et al. (1990) and Okabe et al. (1990) describe additional G-CSF muteins with enhanced biological properties. Elliot and Byrne (1995) have described mutants of EPO with enhanced biological activities.

        Methods of producing and purifying the Ig fusion proteins are also provided. Such methods

25   are generally accomplished by obtaining a transformed or transfected host cell with at least one nucleic acid encoding a growth factor or a cytokine that is not IL-10 and an Ig domain. The host cell is cultured until the desired fusion protein is expressed, followed by the purification or isolation of the Ig fusion protein by any means known in the art or as described in the Examples below. In one embodiment, methods of purifying the fusion proteins include obtaining a composition containing

30   recombinantly produced Ig fusion proteins and isolating the desired Ig fusion proteins from various contaminants using column chromatography procedures, for example, a sizing column with a desired molecular weight cutoff. The host cell can be any suitable prokaryotic or eukaryotic cell line known to those skilled in the art, including without limitation, bacterial, insect or mammalian cells. Particularly useful cells are eukaryotic cells, preferably mammalian cells, and more preferably COS or CHO cells.

35   The Ig fusion proteins produced by the present methods can be used for a variety of *in vitro* and *in vivo* uses. The proteins of the present invention can be used for research, diagnostic or therapeutic purposes that are known f r their wildtype, natural, or previously kn wn modified counterparts.

In vitro uses include, for example, the use of the protein for screening, detecting and/or purifying other proteins. The Ig fusion proteins described herein also are useful as diagnostic reagents for identifying cells expressing receptors for EPO, G-CSF and GH, GM-CSF, IL-11, TPO, SCF, Flt3 ligand and alpha, beta and gamma interferon. The Ig fusion proteins also are useful as *in vitro* reagents

5   for studying cell proliferation and differentiation.

The Ig fusion proteins described herein can be used to treat the same human conditions as the non-fused proteins. For example, the EPO-IgG fusion proteins can be used to treat anemia resulting from kidney failure, chemotherapy, radiation and drug complications. The EPO-IgG fusion proteins also can be used to stimulate red blood cell formation in normal individuals who wish to enhance their

10   red blood cell count or hematocrit prior to surgery. The G-CSF-IgG fusion proteins can be used to treat neutropenia resulting from chemotherapy, radiation and drug complications, for mobilization of progenitor cells for collection in peripheral blood progenitor cell transplants and for treatment of severe ·chronic neutropenia. The GH-IgG fusion proteins can be used to treat short stature and cachexia. The IFN-α-IgG fusion proteins can be used to treat viral diseases and cancer. The IFN-β-IgG fusion

15   protein can be used to treat multiple sclerosis, viral diseases and cancer. The GM-CSF-IgG fusion proteins can be used to treat neutropenia resulting from chemotherapy, radiation and drug complications, for mobilization of progenitor cells for collection in peripheral blood progenitor cell transplants, for treatment of severe chronic neutropenia and as an adjuvant for cancer vaccines. The SCF-IgG and Flt3 ligand-IgG fusion proteins can be used to mobilize hematopoietic progenitor cells

20   for collection in peripheral blood progenitor cell transplants. The IL-11-IgG and TPO-IgG fusion proteins can be used for treating thrombocytopenia.

The Ig fusion proteins described herein will possess longer circulating half-lives in patients, which will allow the fusion proteins to be administered less frequently and/or in effective lower doses than the non-fused proteins. Typically, GH and G-CSF are administered by daily injections and EPO is

25   administered by thrice weekly injections. One skilled in the art can readily determine the appropriate dose, frequency of dosing and route of administration. Factors in making such determinations include, without limitation, the nature and specific activity of the protein to be administered, the condition to be treated, potential patient compliance, the age and weight of the patient, and the like. The compounds of the present invention can also be used as delivery vehicles for enhancement of the circulating half-life

30   of the therapeutics that are attached or for directing delivery to a specific target within the body.

Pharmaceutical compositions containing the Ig fusion proteins of the present invention in a pharmaceutically acceptable carrier are also provided. Those skilled in the art can readily identify such pharmaceutically acceptable carriers, which include without limitation physiological saline, Ringer's solution and the like. Other agents can also be included in the pharmaceutical compositions, including

35   drugs, reagents, and therapeutic agents.

Detailed methods for constructing the Ig fusion proteins of the present invention are provided in the following examples, which are intended to illustrate without limiting the present invention.

Example 1

C nstruction of Growth Factor/Cyt kine-IgG Gene Fusions.

A. General Strategy.

Growth factor/cytokine (GF)–IgG gene fusions were constructed as described below. The
5 general strategy employed for these constructions is outlined here and the specifics of individual
cloning steps are detailed below. Cloning of the IgG4-C_H coding sequence involved additional
variations to the general strategy and these variations are described below. The human growth factor
genes (GH, EPO and G-CSF) were cloned as cDNAs from various RNA sources detailed below. PCR
primers used in these clonings added an optimized Kozak sequence (GCCACC; Kozak, 1991) and a
10 *Hind* III restriction site to the 5' end of each these clones and a portion of a peptide linker (ser-gly-gly-
ser) (SEQ.ID.NO.1) terminating in a *Bam* HI restriction site, to the 3' end of each of these clones. The
growth factor genes were cloned as *Hind* III – *Bam* HI fragments into the mammalian cell expression
vector pCDNA3.1(+) (Invitrogen, Inc., San Diego, CA) and sequenced. In parallel, IgG coding
sequences (IgG1-Fc, IgG1-C_H, IgG4-Fc, IgG4-C_H) were cloned from cDNAs generated from human
15 leukocyte RNA. PCR forward primers used in these clonings incorporated a portion of a peptide linker
(gly-ser-gly-gly-ser)(SEQ.ID.NO.2) containing a *Bam* HI restriction site at the 5' end of each of these
clones. The reverse PCR primers were designed to anneal to the 3' untranslated regions of the IgG1
and IgG4 mRNAs (about 40 bp downstream of the translational stop codon) and included an *Xba* I
restriction site. The IgG coding sequences were cloned into pCDNA3.1(+) as *Bam* HI – *Xba* I
20 fragments and confirmed by DNA sequencing. The fusion genes were then constructed by excising the
IgG coding sequences as *Bam* HI – *Xba* I fragments and cloning these fragments into the pCDNA::GF
recombinant plasmids that had been cut with *Bam* HI and *Xba* I. In the resulting pCDNA3.1 constructs
the fusion genes are transcribed by the strong cytomeglovirus immediate early promoter present in
pCDNA3.1(+) upstream of the cloned fusion gene. Ligation of the two fragments through the *Bam* HI
25 site within the linker sequence results in a seven amino acid linker (ser-gly-gly-ser-gly-gly-
ser)(SEQ.ID.NO.3) at the fusion junction.

B. Cloning of Growth Hormone, EPO and G-CSF

1. Cloning of human Growth Hormone : A cDNA encoding human Growth Hormone (GH) was
amplified from human pituitary single-stranded cDNA (CLONTECH, Inc., Palo Alto, CA), using the
30 polymerase chain reaction (PCR) technique and primers BB87 (5>
GCAAGCTTGCCACCATGGCTACAGGCTCCCGGACG 3)(SEQ.ID.NO.4) and BB88 (5>
CGCGGATCCTCCGGAGAA GCCACAGCTGCCCTCCAC>3)(SEQ.ID.NO.5). Primer BB87
anneals to the 5' end of the coding sequence for the hGH secretion signal, whereas the reverse primer,
BB88, anneals to the 3' end of the GH coding sequence. The resulting ~ 680 bp PCR product was
35 digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector (Invitrogen, Inc.,
Carlsbad, CA) that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel
purified. A clone with the correct DNA sequence (Martial et al., 1979; Roskam and Rougeon, 1979;
Seeburg, 1982; DeNot et al., 1981) was designated pCDNA3.1(+)::GHfus or pBBT159.

2. Cloning of human Erythropoietin . A cDNA encoding human erythropoietin (EPO) was cloned by PCR using forward primer BB45 (5> CCCGGAT CCATGGGGGTGCACGAATGTCCTG >3)(SEQ.ID.NO.6) and reverse primer BB47 (5> CCCGAATTCTATGCCCAGGT GGACACACCTG >3)(SEQ.ID.NO.7). BB45 anneals to the DNA sequence encoding the initiator methionine and amino terminal portion of the EPO signal sequence and contains a *Bam* HI site for cloning purposes. BB47 anneals to the 3' untranslated region of the EPO mRNA immediately downstream of the translational stop signal and contains an *Eco* RI restriction site for cloning purposes. Total RNA isolated from the human liver cell line Hep3B was used in first strand synthesis of single-stranded cDNA for PCR. For preparation of total cellular RNA, Hep3B cells (available from the American Type Culture Collection, Rockville, MD) were grown in Delbecco's Modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS). EPO expression was induced by treating the cells for 18 h with 130 µM Deferoxamine or 100 µM cobalt chloride (Wang and Semenza, 1993). RNA was isolated from the cells using an RNeasy Mini kit (Qiagen, Santa Clarita, CA), following the manufacturer's directions. Approximately 320 µg of total RNA was isolated from 1.4×10^7 cells treated with cobalt chloride and 270 µg of total RNA isolated from 1.4×10^7 cells treated with Deferoxamine.

First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corporation (Indianapolis, IN) and random hexamers were used as the primer. Subsequent PCR reactions using the products of the first strand syntheses as templates were carried out with primers BB45 and BB47. The expected approximately (~) 600 bp PCR product was observed when reaction products were run out on an agarose gel. Both RNA preparations yielded an EPO PCR product. The PCR product was digested with *Bam* HI and *Eco* RI and cloned into vector pUC19 that had been cut with *Bam* HI and *Eco* RI and treated with alkaline phosphatase. DNA sequencing identified a clone containing the correct coding sequence for the EPO gene. This plasmid was designated pBBT131.

Plasmid pBBT131was used as template in a PCR reaction with primers BB89 (5>CGCAAGCTTGCCACCATGGGGGGTGC ACGAATGTCCT >3)(SEQ.ID.NO.8) and BB90 (5>CGCGGATCCTCCGGATCTGTCCCCTGTCCTGCAGGC >3)(SEQ.ID.NO.9) to construct a modified EPO cDNA suitable for fusion with IgG genes. Primer BB89 anneals to the 5' end of the coding sequence for the EPO secretion signal and the reverse primer, BB90, anneals to the 3' end of the EPO coding sequence. The resulting ~ 610 bp PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Lin et al., 1985) was designated pCDNA3.1(+)::EPOfus or pBBT176.

Similar procedures can be used to create modified EPO cDNAs in which Arg166 is deleted. In this case a primer with the sequence (5>CGCGGATCCTCCGGATCTGTCCCCTGTCCTG CAGGCCTC>3)(SEQ.ID.NO.10) can be used in place of primer BB90.

3. Cl ning of human Granulocyte Colony-Stimulating Fact r. A cDNA encoding human Granulocyte Colony Stimulating Factor (G-CSF) was amplified by PCR from total RNA isolated from

the human bladder carcinoma cell line 5637 (available from the American Type Culture Collection, Rockville, MD). The cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. RNA was isolated from the cells using an RNeasy Mini RNA isolation kit purchased from Qiagen, Inc. (Santa Clarita,

5   CA) following the manufacturer's directions. Approximately 560 μg of total RNA was isolated from 4.5 x10^7 cells. First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp (Indianapolis, IN) and random hexamers were used as the primer. Subsequent PCR reactions using the products of the first strand synthesis as template were carried out with forward primer BB91

10   (5>CGCAAGCTTGCCACCATGGCTGGACC TGCCACCCAG>3) (SEQ.ID.NO.11) and reverse primer BB92 (5>CGCGGATCCTCCGGAGGGCTGGGCAAGGTGGCGTAG >3)(SEQ.ID.NO.12). Primer BB91 anneals to the 5' end of the coding sequence for the G-CSF secretion signal and the reverse primer, BB92, anneals to the 3' end of the G-CSF coding sequence. The resulting ~ 640 bp PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+)

15   vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Souza et al., 1986; Nagata et al., 1986a,b) was designated pCDNA3.1(+)::G-CSFfus or pBBT165.

C. Cloning of IgG coding sequences.

20   1. Cloning of IgG1-Fc coding sequences. A cDNA encoding IgG1-Fc (hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH, Inc., Palo Alto, CA) by PCR using primers BB83 (5>CGCGGATCCG GTGGCTCAGAGCCCAAATCTTGTGACAAAACT >3)(SEQ.ID.NO.13) and BB82 (5>CGCTCTAG AGGTACGTGCCAAGCA TCCTCG>3)(SEQ.ID.NO.14). Forward primer BB83 anneals to the 5' end of the coding sequence of

25   the hinge domain of IgG1, whereas the reverse primer BB82 anneals to the 3' untranslated region of IgG1 and IgG4 mRNA ~ 45 bp downstream of the translational stop codon. The IgG1 and IgG4 sequences are identical over the 21 bp segment to which BB82 anneals. The ~ 790 bp PCR product was digested with *Bam* HI and *Xba* I, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Bam* HI and *Xba* I, alkaline phosphtase treated, and gel purified. Two clones were

30   sequenced but each contained a single base pair substitution that resulted in an amino acid substitution mutation. Otherwise the sequences matched the published human IgG1 genomic DNA sequence (Ellison et al., 1982). The relative positions of the mutations in the two clones allowed us to use convenient unique restriction sites (*Sac* II in the CH2 domain of IgG1 and *Bgl* II in the pCDNA3.1(+) vector) to construct a full length IgG1-Fc clone in pCDNA3.1(+) via *in vitro* recombination. The

35   resulting clone, which had the correct IgG1-Fc sequence, was designated pCDNA3.1(+)::fusIgG1-Fc or pBBT167.

2. Cloning f IgG4-Fc coding sequences. A cDNA encoding IgG4-Fc (hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using primers BB84

(5>CGCGGATCCGG TGGCTCAGAGTCCAAATATGGTCCCCCATGC >3)(SEQ.ID.NO.15) and BB82 (5>CGCTCTAG AGGTACGTGCCAAGCA TCCTCG>3)(SEQ.ID.NO.14). Forward primer BB84 anneals to the 5' end of the coding sequence of the hinge domain of IgG4. The reverse primer BB82 is described above. The ~ 790 bp PCR product was digested with *Bam* HI and *Xba* I and cloned into pCDNA3.1(+) that had been similarly digested. A clone with the correct DNA sequence (Ellison et al., 1981) was designated pCDNA3.1(+)::fusIgG4-Fc or pBBT158.

3. **Cloning of IgG1-C$_H$ coding sequences.** A cDNA encoding IgG1-C$_H$ (CH1-hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using BB81 (5>CGCGGATCC GGTGGCTCAGCCTCCACCAAGGGCCCATC>3)(SEQ.ID.NO.16) and BB82(5>CGCTCTAGAGGTACGTGCCAAGC ATCCTCG>3)(SEQ.ID.NO.14). Forward primer BB81 anneals to the 5' end of the coding sequence of the CH1 domain of IgG1 and IgG4. The sequences at the 5' end of the CH1 domains of these two exons are almost identical: 19/20 nucleotides match. The reverse primer, BB82, is described above. The ~ 1080 bp PCR product was digested with *Bam* HI and *Xba* I, gel purified and cloned into pCDNA3.1(+) that had been digested similarly. These primers in principle could amplify both IgG1 and IgG4 sequences. Since IgG1 is much more abundant in serum than IgG4 (Roitt et al., 1989) we expected that most clones would encode IgG1. The first two clones sequenced were IgG1 but each contained a single base pair substitution that resulted in an amino acid substitution mutation. Otherwise the sequences obtained matched the published human IgG1 genomic DNA sequence (Ellison et al., 1982). The relative positions of the mutations in the two clones allowed us to use convenient unique restriction sites (*Age* I in the CH1 domain of IgG1 and *Bst* BI in the pCDNA3.1(+) vector) to construct a full length IgG1-C$_H$ clone in pCDNA3.1(+) via *in vitro* recombination. A clone with the correct IgG1-C$_H$ sequence was designated pCDNA3.1(+)::fusIgG1-C$_H$ or pBBT166.

4. **Cloning of IgG4-C$_H$ coding sequences.** The near identity of the DNA sequences encoding the 5' ends of the IgG1 and IgG4 CH1 domains and the relatively low abundance of the IgG4 mRNA led to an alternative strategy for cloning the IgG4-C$_H$ coding sequences. PCR-based site directed mutagenesis was used to change the DNA sequence of the cloned IgG1 CH1 domain to match the amino acid sequence of the IgG4 CH1 domain. The CH1 domains differ at only 8 of 98 amino acids and these positions are clustered, so that one round of PCR using two mutagenic oligos can convert the IgG1 CH1 sequence into the IgG4 CH1 sequence. A second round of PCR added the *Bam* HI site and linker sequence to the 5' end of the IgG4 CH1 and 21 bp of sequence from the IgG4 Hinge domain to the 3' end. The technique of "gene splicing by overlap extension" (Horton et al., 1993; Innis et al., 1990) was then employed to recombine the engineered IgG4 CH1 domain with the IgG4 Fc (Hinge-CH2-CH3) sequence. In this technique two separate fragments sharing a segment of identical sequence, the "overlap", at one end are extended through the annealed overlap regions in a PCR reaction as diagrammed in Figure 1.

To construct the IgG4 CH1 sequence, mutagenic primers BB119 (5> TCCACCAAG GGCCCATCCGT CTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGC ACAGCC>3)(SEQ.ID.NO.17) and BB120 (5> TCTCTTG TCCACCTTGGTGTTGCTGGGCTTGTGATC TACGTTGCAGGTGTAGGTCTTCGTGCCCAA >3)(SEQ.ID.NO.18) were used in PCR reactions with pBBT166, which carries the cloned IgG1-C$_H$ sequence as described above. Forward primer BB119 anneals to the sequence encoding amino acids 2 through 23 of the CH1 domain and encodes 4 amino acid substitutions: S14C, K16R, G20E and G21S. Reverse primer BB120 anneals to the sequence encoding amino acids 76 through 97 of the CH1 domain and encodes 4 additional amino acid substitutions: Q79K, I82T, N86D and K97R. The ~ 290 bp product of this PCR reaction was gel purified and used as template in a PCR reaction with primers BB81(see above; **Example 1, C.3**) and BB121(5>TGGGGGACCATATTTG GACTCAACTCTCTTGTCCACCTT >3)(SEQ.ID.NO.19). Reverse primer BB121 anneals to the 3' end of the CH1 domain of IgG4, adds amino acid 98 of the CH1 domain and 21 bp extending into the Hinge domain of IgG4. The ~ 330 bp product of this reaction was gel purified and used as one of the template molecules in the PCR splicing reaction. The other template for the splicing reaction was generated by PCR of the cloned IgG4-Fc sequence of pBBT158 (described above, **Example 1, C.2.**) with primers BB84 and BB82 which amplify the IgG4 Fc domain as described above. The resulting ~ 790 bp product consists of the IgG4 hinge-CH2-CH3 sequence. This fragment was gel purified and used as one of the template molecules in the PCR splicing reaction. This reaction employed the primers BB81 and BB82 and generated a full-length "spliced" product of ~ 1075 bp. To minimize the DNA sequencing required to confirm this product, the PCR fragment was digested with *Bam* HI and *Sac* II and the ~ 530 bp fragment (containing the complete CH1 and hinge domains and a portion of the CH2 domain) was cloned into pBC-SK+ (Stratagene) for sequencing. The sequence of the *Bam* HI – *Sac* II fragment was confirmed for one clone which was the designated pBBT182. The *Bam* HI – *Sac* II fragment of pBBT182 was then used convert the GF–IgG4-Fc clones to full length GF–IgG4-C$_H$ clones as detailed below.

D. Construction of Growth Hormone-, EPO- and G-CSF–IgG Fusions.

Most (9/12) of the EPO-, G-CSF- and GH-IgG gene fusions were generated by excising the IgG coding sequences cloned in pCDNA3.1(+) as *Bam* HI – *Xba* I fragments and cloning these fragments into the pCDNA::[GF] recombinant plasmids which had been cut with *Bam* HI and *Xba* I. The fusions of the three growth factor genes to IgG4-C$_H$ were constructed by excising the ~ 530 bp *Bam* HI– *Sac* II fragment of pBBT182 and replacing the ~ 240 bp *Bam* HI- *Sac* II fragments of the three pCDNA::[GF]-IgG4-Fc clones with this ~530 bp *Bam* HI-*Sac* II fragment. The resulting plasmids and the GF-IgG fusion proteins they encode are listed in Table 1.

E. G-CSF (C17S)-IgG Fusion Proteins

G-CSF-IgG fusions in which cysteine-17 f G-CSF is replaced by serine [G-CSF (C17S)] can be constructed by PCR mutagenesis using the following tw   ligonucleotide primers: C17S-F

(5'TTCCTGCTCAAGTCCTTAGAGCAAGTG-3')(SEQ.ID.No.20) and C17S-R

(5'CACTTGCTCTAAGGACTTGAGCAGGAA-3')(SEQ.ID.NO.21). For example, a G-CSF (C17S)-

IgG1-Fc fusion can be constructed by performing two separate PCR reactions using pBBT174 as template.

One reaction can use oligo C17S-F in combination with BB82. The second PCR reaction can use ligo

C17S-R in combination with BB91. The PCR products of these reactions can be gel-purified, mixed and

reamplified with BB82 and BB91. The ~ 1300 bp product can be digested with *Hind*III and *Pfl*MI, the

*Hind*III/*Pfl*MI band gel-purified, and cloned into similarly cut plasmid pBBT174. After transformation of

E. coli DH5α, a plasmid clone containing the C17S mutation can be identified by DNA sequencing.

Similar mutagenesis procedures can be used to change cysteine-17 to another amino acid by substituting the

appropriate nucleotides (sense and antisense nucleotides encoding the desired amino acid) for the

underlined nucleotides in oligos C17S-F and C17S-R.

Example 2

Expression and Purification of GF-IgG Fusion Proteins

A. Small Scale Transfection of COS Cells

Expression and bioactivity of the GF-IgG fusion proteins were assessed initially by small-

scale transfection of COS cells. Endotoxin-free plasmid DNAs were prepared using an "Endo-Free

Plasmid Purification Kit" (Qiagen, Inc.) according to the vendor protocol and used to transfect COS-1

cells (available from the American Type Culture Collection, Rockville, MD). The COS-1 cells were

grown in Delbecco's Modified Eagle's Media supplemented with 10% FBS, 50units/ml penicillin,

50µg/ml streptomycin and 2mM glutamine (COS cell growth media). Initial transfection experiments

were carried out in Costar 6 well tissue culture plates (VWR Scientific) using the following protocol.

Briefly, 2-3 x 10^5 cells were seeded into each well in 2 ml of COS cell growth media and allowed to

incubate overnight at 37°C and 5% CO_2 by which time the cells had reached 50-60% confluency. For

each well, 0.8 µg of plasmid DNA was complexed with 6 µl of LipofectAMINE reagent (Gibco BRL,

Gaithersburg, MD) in 186 µl of OPTI-MEM I Reduced Serum Medium (Gibco BRL, Gaithersburg,

MD) for 30-45 minutes at room temperature. COS-1 cells were washed one time with 2ml of OPTI-

MEM I per well and then 1.8 ml of OPTI-MEM I was added to each well. The complex mixture was

then added to the well and left at 37°C, 5% CO_2 for approximately 4-5 hours. After the incubation

period, the mixture was replaced with 2 ml of COS cell growth media per well and left overnight at

37°C, 5% CO_2. The next day the cells were washed twice with 2ml of DMEM (no additives) per well.

Following the wash steps, 2 ml of serum-free COS cell growth media was added to each well and the

cells left at 37°C, 5% CO_2. Conditioned media containing the GF-IgG-fusion proteins were harvested

after 72 hours and analyzed by SDS-PAGE and Western blot to confirm expression of the GF-IgG-

fusion proteins. The parent plasmid, pCDNA 3.1(+) (Invitrogen) was used as a negative control.

Transfection efficiency was estimated to be ~15%, using pCMVβ (Clontech), which expresses *E. coli*

β-galactosidase. Transfected cells expressing β-galactosidase were identified using a β-Gal Staining

Set (Boehringer Mannheim, Indianapolis, IN).

Samples of the c nditioned media were prepared in SDS-PAGE sample buffer with the addition of 1% β-mercapt ethanol (BME) when desirable and electroph resed on precast 14% Tris-glycine polyacrylamide gels (Novex). Western blots using appropriate antisera demonstrated expression of all of the GF-IgG fusion proteins. The GH-IgG fusion proteins were detected using a
5   polyclonal rabbit anti-synthetic-hGH antiserum (kindly provided by Dr. A.F. Parlow and the National Hormone and Pituitary Program). The EPO- and G-CSF-IgG fusion proteins were detected using polyclonal antisera purchased from R&D Systems (Minneapolis, MN). Serial dilutions of the conditioned media were analyzed in the appropriate *in vitro* bioassays described later. These assays demonstrated significant activity in the conditioned media.

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B. Large Scale Transfection of COS-1 Cells

Large scale transfections were carried out using Corning 100 mm tissue culture dishes or Corning T-75 tissue culture flasks (VWR Scientific). For 100 mm dishes, 1.6 x 10⁶ cells were plated in 10 ml of COS cell growth media per dish and incubated at 37°C, 5% CO_2 overnight. For each 100 mm
15   dish, 6.7 µg of endotoxin-free plasmid DNA was complexed with 50 µl of LipofecAMINE reagent in 1.5 ml of OPTI-MEM I for 30-45minutes at room temperature. The COS-1 cells were washed one time with 10 ml OPTI-MEM per dish and then replaced with 6.6 ml of OPTI-MEM I. Following complex formation, 1.67 ml of the complex was added to each dish and left at 37°C, 5% CO_2 for 4-5 hours. After the incubation period, the reaction mixture was replaced with 10 ml of serum containing COS
20   cell growth media per dish and left at 37°C, 5% CO_2 overnight. The next day the cells were washed twice with 10 ml of DMEM (no additives) per dish. Following the wash steps, 10ml of serum-free COS cell growth media was added to each dish and incubated at 37°C, 5% CO_2. Conditioned media were harvested routinely every three days (on days 3, 6, 9 and 12) and fresh serum-free COS cell growth media added to the cells. Transfections in T-75 culture flasks were identical to the 100mm dish
25   protocol with the following exceptions: Cells were plated at 2 x 10⁶ cells per flask and 9.35 µg of endotoxin-free plasmid DNA was complexed with 70 µl of LipofectAMINE reagent in 2.1ml of OPTI-MEM I for each T-75 flask. Following complex formation, 2.3 ml of the complex was added to each flask containing 7.7 ml of OPTI-MEM I. Transfection efficiency was determined to be ~15% using pCMVβ and staining for β-galactosidase expression as decribed earlier. The 12 plasmids listed in Table
30   1 were transfected into COS-1 cells using the large-scale format to generate protein for purification. The conditioned media were clarified by centrifugation and stored at -80°C for later purification. Western blots were used to confirm expression of the IgG-fusion proteins.

C. Purification of GF-IgG-Fusion Proteins

35   Approximately 300 ml of transfected COS-1 cell conditioned media for each IgG-fusion protein was pooled and concentrated using an Ultrafiltration cell and either a YM3 or YM30 DIAFLO Ultrafiltration membrane (Amicon, Beverly, MA). Concentrated pools were then loaded nto a 1ml Pharmacia HiTrap rec mbinant Protein A column previ usly equilibrated with 20 mM NaPhosphate pH 7.0. The column was washed with 20 mM NaPhosphate until the A_{280} had reached baseline.

Bound protein was eluted with 100 mM NaCitrate pH 3.0 and collected into sufficient 1M Tris pH 9.0 to achieve a final pH of approximately 7.0. Each fusion protein was purified using a dedicated column to avoid any possibility of cross-contamination. All of the IgG fusion proteins chromatographed similarly, yielding a single peak in the elution step. Column fractions were analyzed using 8-16%

5   precast Tris-glycine SDS-PAGE and fractions enriched for the IgG-fusion protein were pooled. Protein concentrations of the pooled fractions were determined by Bradford assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin (BSA) as the standard.

The purified GF-IgG fusion proteins were analyzed by SDS-PAGE under reducing and non-reducing conditions and stained with Coomassie blue. All of the GF-IgG fusion proteins were

10   recovered principally as disulfide-linked dimers (typically, >90% of the recovered IgG fusion proteins were dimeric). The apparent molecular weights of the proteins ranged from 115-190 kDa kDa under non-reducing conditions (disulfide-linked dimers) and 50-75 kDa under reducing conditions (monomers), and were largely consistent with the molecular weights predicted in Table 1. The size differences between monomers and dimers allowed monomers and dimers to be easily distinguished by

15   SDS-PAGE. The apparent molecular weights of the monomeric and dimericGH-IgG-Fc fusion proteins were approximately 50 kDa and 115 kDa, respectively. The apparent molecular weights of the monomeric and dimericG-CSF-IgG-Fc fusion proteins also were approximately 50 kDa and 115 kDa, respectively.   The apparent molecular weights of the dimeric IgG-C_H fusions were larger than expected: approximately 160 kDa for the GH-IgG-C_H and G-CSF-IgG-C_H fusions and approximately

20   180 kDa for the EPO-IgG-C_H fusions. The EPO-IgG-C_H fusion monomer also was larger than expected, possessing an apparent molecule weight of approximately 74 kDa. The molecular weights of the EPO-IgG-Fc fusion proteins also were larger than predicted (apparent monomer and dimer molecular weights of 62 kDa and 130 kDa, respectively). The larger than expected sizes of the EPO-Ig fusion proteins presumably are due to extensive glycosylation of the EPO domain. Monomeric

25   fusion proteins were present in all of the purified protein samples, but were more abundant with the IgG4 fusion proteins. The sizes of the major IgG fusion protein bands were different from the molecular weights of bovine IgG, indicating that the proteins purified were not contaminating bovine IgGs from serum used in the experiments. The major IgG fusion protein bands also reacted with antisera specific for GH, EPO and G-CSF in Western blots of the samples. Purity of the IgG fusion

30   proteins was estimated to be at least 90% from Coomassie blue staining of the gels.

All of the GF-IgG-C_H fusions contained a large aggregate that migrated at the top of the gel when the samples were analyzed under non-reducing conditions. This aggregate disappeared when the samples were analyzed under reducing conditions and the amount of protein at the molecular weight of the major GF-IgG-C_H bands seemed to increase proportionately. The aggregates also reacted with

35   antisera specific for the various growth factors. These data suggest the aggregates are disulfide-linked multimers of the GF-IgG-C_H fusion proteins. Under reducing SDS-PAGE conditions, all of the GF-IgG-C_H fusions show a diffuse band approximately 20 kDa larger than the main GF-IgG-C_H band. This band reacted with antisera against the growth factors and may be related to the aggregates.

Table 1 Predicted M lecular Weights and Recoveries of GF-IgG Fusion Proteins

Expression Plasmid	IgG-Fusion Protein	Predicted Molecular Weight (kDa)[1]	
		Monomer	Dimer
PBBT 171	GH-IgG1-C_H	58,706	117,412
PBBT 172	GH-IgG1-Fc	48,693	97,386
PBBT183	GH-IgG4-C_H	58,541	117,082
PBBT 163	GH-IgG4-Fc	48,365	96,730
PBBT 173	G-CSF-IgG1-C_H	55,564	111,128
PBBT 174	G-CSF-IgG1-Fc	45,551	91,102
PBBT 184	G-CSF-IgG4-C_H	55,399	110,798
PBBT 175	G-CSF-IgG4-Fc	45,222	90,444
PBBT 179	EPO-IgG1-C_H	54,972	109,944
PBBT 180	EPO-IgG1-Fc	44,960	89,920
PBBT 185	EPO-IgG4-C_H	54,808	109,616
PBBT 181	EPO-IgG4-Fc	44,632	89,264

[1] Does not include molecular weight contributions due to of glycosylation.

Example 3

In Vitro **Bioactivities of Purified GF-IgG Fusion Proteins**

A. General Strategy

Cell proliferation assays were developed to measure *in vitro* bioactivities of the IgG fusion proteins. The assays measure uptake and bioreduction of the tetrazolium salt MTS [3-(4,5-dimethylthiazol-2-yl)-5-3-carboxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium]. In the presence of an electron coupler such as phenazine methosulfate (PMS), MTS is converted to a formazan product that is soluble in tissue culture media and can be measured directly at 490 nm. Cell number is linear with absorbance values up to about 2. For EPO and G-CSF we were able to use existing cell lines to develop the bioassays. For GH, we needed to create a cell line that proliferates in response to GH. Such a cell line was created by stably transforming a murine leukemia cell line with a rabbit GH receptor.

In general, the bioassays were set up by washing the appropriate cells three times with media (no additives) and resuspending the cells at a concentration of $0.7-1x10^5$/ml in media with additives (media used for each cell line is given below). Fifty µl ($3.5-5x10^3$ cells) of the cell suspension was aliquotted per test well of a flat bottom 96 well tissue culture plate. Serial dilutions of the protein samples to be tested were prepared in serum-containing media. Fifty µl of the diluted protein samples were added to the test wells and the plates incubated at 37^0C in a humidified 5% CO_2 tissue culture incubator. Protein samples were assayed in triplicate wells. After 60-72 h, 20 µl of an MTS/PMS mixture (CellTiter 96 AQueous One Solution, Promega Corporation, Madison, WI) was added to each well and the plates incubate at 37^0C in the tissue culture incubator for 1-4 h. Absorbance of the wells

20

was read at 490 nm using a microplate reader. Control wells contained media but no cells. Mean absorbance values f r the triplicate control wells were subtracted from mean values obtained for the test wells. EC_{50}s, the amount of protein required for half maximal stimulation, was calculated for each sample and used to compare bioactivities of the proteins. Non-glycosylated m lecular weights were

5　used in the molar ratio calculations for consistency. Non-glycosylated molecular weights of 18,936, 18,987 and 22,129 were assumed for EPO, G-CSF and GH, respectively. Monomer molecular weights were used in the calculations for the IgG fusion proteins. Using molecular weights of the monomer fusion proteins estimated from SDS gels (50-70 kDa) and glycosylated molecular weights of 35kDa for EPO and 19.7 kDa for G-CSF (GH is not glycosylated) gave similar activity ratios.

10

B. Bioactivities of EPO-IgG Fusion Proteins

The human UT7/epo cell line was obtained from Dr. F. Bunn of Harvard Medical School, Boston, MA. This cell line proliferates in response to EPO and is dependent upon EPO for cell survival (Boissel et al., 1993). The cells were maintained in Iscove's Modified Delbecco's Media (IMDM)

15　supplemented with 10% FBS, 50 units/ml penicillin, 50 µg/ml streptomycin and 1 unit/ml recombinant human EPO (CHO cell-expressed; R&D Systems). Bioassays were performed in cell maintenance media using the procedures described above. Serial dilutions of recombinant CHO cell-expressed human rEPO (R&D Systems) were analyzed in parallel.

The UT7/epo cell line shows a strong proliferative response to rEPO, as evidenced by a dose-

20　dependent increase in cell number and absorbance values. In the absence of rEPO, the majority of UT7/epo cells die, giving absorbance values less than 0.1. Commercial CHO cell-expressed rEPO had a mean EC_{50} of approximately 0.6 ng/ml in the bioassay (Table 2). This value agrees with EC_{50} values reported in the R&D Systems specifications (0.05 – 0.1 unit/ml or approximately 0.4-0.8 ng/ml). The EPO-IgG1-Fc and IgG4-Fc fusion proteins had identical EC_{50}'s of approximately 1.3 ng/ml in the

25　bioassay (Table 2). On a molar basis, the EC_{50}s of CHO-cell expressed rEPO and the EPO-IgG-Fc fusions were indistinguishable (approximately 30 pM; Table 2). The EPO-IgG1-C_H fusion protein had an EC_{50} of 3.1 ng/ml or 60 pM (Table 2), which represents an approximate 2-fold reduction in specific activity relative to the EPO-IgG-Fc fusion proteins and non-fused rEPO. The EPO-IgG4-C_H fusion protein had a mean EC_{50} of 2.05 ng/ml.

30

Table 2. Bioactivities of EPO-IgG Fusion Proteins

Clone	Protein	EC_{50} Range (ng/ml)[1]	Mean EC_{50}	
			ng/ml	pM
-	RhEPO (CHO)	0.52, 0.55, 0.60	0.56	30
pBBT180	EPO-IgG1-Fc	1.1, 1.2, 1.5	1.27	28
pBBT181	EPO-IgG4-Fc	1.1, 1.2, 1.5	1.27	29
pBBT179	EPO-IgG1-C_H	2.9, 3.0, 3.5	3.13	57
pBBT185	EPO-IgG4-C_H	2.0, 2.1	2.05	37

[1]Data from individual experiments

C. Bioactivities of G-CSF-IgG Fusi n Proteins

The murine NFS60 cell line was obtained from Dr. J. Ihle f the University of Tennessee Medical School, Memphis Tennessee. This cell line proliferates in response to human or mouse G-CSF or IL-3 (Weinstein et al., 1986). The cells were maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/ml penicillin, 50 µg/ml streptomycin and 17-170 units/ml mouse IL-3 (R&D Systems). Assays were performed in RPMI 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin using the procedures described above. Serial dilutions of recombinant human G-CSF (*E. coli*-expressed; R&D Systems) were analyzed in parallel.

The NFS60 cell line shows a strong proliferative response to rhG-CSF, as evidenced by a dose-dependent increase in cell number and absorbance values. rhG-CSF had a mean EC_{50} of 18 pg/ml in the bioassay (Table 3). This value agrees with the EC_{50} value reported in the R&D Systems specifications (10-30 pg/ml). The G-CSF-IgG1-Fc and G-CSF-IgG4-Fc fusion proteins had mean EC_{50}'s of 38 and 57 pg/ml, respectively, in the bioassay (Table 3). On a molar basis, the EC_{50}s of rhG-CSF and the G-CSF-IgG1-Fc fusions were similar (approximately 0.9 pM; Table 3), whereas the EC_{50} of the G-CSF-IgG4-Fc fusion protein was reduced slightly (1.25 pM). The G-CSF-IgG1-C_H fusion protein had a mean EC_{50} of 182 pg/ml or 3.2 pM (Table 3), which represents an approximate 3-fold reduction in specific activity relative to G-CSF-IgG1-Fc fusion protein and non-fused rhG-CSF. The G-CSF-IgG4-C_H fusion protein had a mean EC_{50} of 182 pg/ml or 3.9 pM (Table 3)

Table 3

Bioactivities of G-CSF- IgG Fusion Proteins Clone	Protein	EC_{50} Range (pg/ml) [1]	Mean EC_{50}	
			pg/ml	pM
-	rhG-CSF	17, 18, 18	17.7	0.93
PBBT174	G-CSF-IgG1-Fc	34, 39, 42	38.3	0.84
PBBT175	G-CSF-IgG4-Fc	50, 59, 61	56.7	1.25
PBBT173	G-CSF-IgG1-C_H	160, 190, 195	182	3.2
PBBT184	G-CSF-IgG4-C_H	200, 230	215	3.9

[1] Data from individual experiments

D. Bioactivities of GH-IgG Fusion Proteins:

The GH-R4 cell line is described in PCT/US98/14497 and PCT/US00/00931. This cell line is a derivative of the murine FDC-P1 cell line that is stably transformed with the rabbit GH receptor. This cell line proliferates in response to hGH. The cell line is maintained in RPMI 1640 media containing 10% horse serum, 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine, 400 µg/ml G418 and 2-5 nM pituitary hGH. GH-IgG fusion protein samples were assayed as described in PCT/US98/14497 and PCT/US00/00931, incorporated herein by reference, using RPMI media supplemented with 10% horse serum, 50 units/ml penicillin, 50 µg/ml streptomycin and 400 µg/ml G418. All assays included a human pituitary GH standard. Pituitary GH stimulates proliferation of

GH-R4 c lls, with an EC_{50} f 0.75-0.85 ng/ml (0.03-0.04 nM), similar to what has been reported in the literature (Rowlinson et al., 1995).

All of the GH-IgG fusion proteins tested stimulated proliferation of GH-R4 cells (Table 4). The GH-IgG1-Fc fusion protein was the most active, possessing an EC_{50} of 7-8 ng/ml (~0.16 nM). The
5   GH-IgG4-Fc fusion protein was about two-fold less active than the IgG1 fusion protein, with a mean EC_{50} of 17 ng/ml (0.35 nM). The GH-IgG1-C_H fusion protein had the lowest activity, with an EC_{50} of 35 ng/ml (0.6nM). On a molar basis, bioactivities of the fusion proteins were reduced 4-fold (GH-IgG1-Fc), 10-fold (GH-IgG4-Fc) and 17-fold (GH-IgG1-C_H) relative to pituitary hGH.

Table 4. Bioactivities of GH-IgG Fusion Proteins

Clone	Protein	EC_{50} Range (ng/ml) [1]	Mean EC_{50}	
			ng/ml	nM
-	Pituitary hGH	0.75, 0.75, 0.85, 0.85	0.8	0.036
PBBT172	GH-IgG1-Fc	6.5, 7.0, 7.8, 9	7.6	0.160
PBBT163	GH-IgG4-Fc	15, 16, 18, 18	17	0.350
PBBT171	GH-IgG1-C_H	28, 32, 40, 40	35	0.600

10   [1] Data from individual experiments

Example 4

Eliminate or Minimize the Linker in the GF-IgG Fusion Proteins

GF-IgG fusion proteins in which the 7 amino acid linker [ser-gly-gly-ser-gly-gly-ser] that
15   fuses the GF to the IgG domain is eliminated (direct fusions) or reduced to 2 to 4 amino acids can be constructed as described below. Similar methods can be used to create linkers shorter than 7 amino acids, to create linkers longer than 7 amino acids and to create linkers containing other amino acid sequences. The experiments described below use IgG1-Fc and EPO and G-CSF as examples, however, similar procedures can be used for other GF or IgG domains, and domains for other IgG subtypes and
20   domains from IgM, IgA,IgD and IgE antibodies. The modified fusion proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in the Examples.

1. Direct Fusions: GF-IgG fusions without a linker can be created using PCR based "gene splicing by overlap extension" as described in Example 1 (Horton et al., 1993). One can generate PCR products
25   consisting of the IgG1-Fc coding sequence with a short 5' extension, consisting of the 3' terminal ~ 15 bp of coding sequence of EPO or G-CSF fused directly to the hinge coding sequence. At the same time one can generate PCR products consisting of the EPO or G-CSF coding sequences with a short 3' extension consisting of the first 15 bp of the hinge coding sequence fused directly to the EPO or G-CSF coding sequence. The growth factor fragments and the IgG1-Fc fragments can then be spliced together
30   via PCR "Sewing" (Horton et al., 1993) to generate direct fusions. These PCR products can be digested with appropriate restriction enzymes to generate relatively small DNA segments that span the fusion point and which can be readily cloned into similarly cut vectors pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc for sequence confirmation and COS cell expression. Cloning these

smaller DNA fragments will minimize the sequencing that will need to be done to confirm the
sequences of the direct fusions.

1. A. EPO-IgG Direct Fusions

5   An EPO-IgG1-Fc direct fusion was created by PCR using plasmid pBBT180 as the DNA
template. One PCR reaction used oligos BB198 (5'-AGGACAGGGGACAGAGAGCCCAAA
TCTTGTGACAAA-3')(SEQ.ID.NO.22) and BB82. The second PCR reaction used oligos BB199
(5'ACAAGATTTGGGCTCTCTGTCCCCTGTCCTGCAGGC-3')(SEQ.ID.NO.23) and BB89. The
products from these PCR reactions were gel-purified, mixed and subjected to a third PCR reaction

10   using oligos BB82 and BB89. The approximate 1300 bp PCR product was gel-purified, digested with
and *Bsr* GI and *Sac* II, and cloned into similarly cut pBBT180 that had been treated with calf intestinal
phosphatase. A clone with the correct insert was identified by DNA sequencing and named pBBT356.

        An EPO-IgG4-Fc direct fusion was created by PCR using plasmid pBBT181 as the DNA
template. One PCR reaction used oligos BB200 (5'-AGGACAGGGGACAGAGAGTCCAAATAT

15   GGTCCCCCA-3') (SEQ.ID.NO.24) and BB82. The second PCR reaction used oligos BB201 (5'-
ACCATATTTGGACTCTCTGTCCCCTGTCCTGCAGGC-3')(SEQ.ID.NO.25) and BB89. The
products from these PCR reactions were gel-purified, mixed and subjected to a third PCR reaction
using oligos BB82 and BB89. The approximate 1300 bp PCR product was gel-purified, digested with
and *Bsr* GI and *Sac* II, and cloned into similarly cut pBBT181 that had been treated with calf intestinal

20   phosphatase. A clone with the correct insert was identified by DNA sequencing and named pBBT357.
A clone with a correct EPO sequence, but a mutation in the IgG4-Fc region (Phe-25 changed to Ser)
was named pBBT 273.

        COS cells were transfected with pBBT273 and the EPO-IgG4-Fc fusion protein purified as
described in **Example 2**. The purified protein, which consisted primarily of a disulfide-linked dimer

25   (>90%), stimulated proliferation of UT7/epo cells with an EC_{50} of 2 ng/ml. The bioassay was
performed as described in **Example 3**.

        An EPO (des-Arg-166)-IgG1-Fc direct fusion can be created as described above for the EPO-IgG1-
Fc direct fusion except that oligonucleotide EPODFE (5'-TGCAGGACAGGGGACGAGCCCAAAT
CTTGTGACAAA-3')(SEQ.ID.NO.26) can be substituted for BB198 and EPODFF (5'-

30   ACAAGATTTGGGCTCGTCCCCTGTCCTGCAGGCCTC-3')(SEQ.ID.NO.27) can be substituted for
BB199. Similarly, EPO(des-Arg-166)-IgG4-Fc direct fusions can be constructed as described above
for EPO-IgG4-Fc fusions except that EPODFG (5'-TGCAGGACAGGGGACGAGTCCAAATAT
GGTCCCCCA-3')(SEQ.ID.NO.28) can be substituted for BB200 and EPODFH (5'-
ACCATATTTGGACTCGTCCCCTGTCCTGCAGGCCTC-3')(SEQ.ID.NO.29) can be substituted for

35   BB201.

1. B. G-CSF Direct Fusions

      A G-CSF-IgG1-Fc direct fusion was created by PCR using plasmid pBBT174 as the DNA template. One PCR reaction used oligos BB202 (5'-CACCTTGCCCAGCCCGAGCCCAAA TCTTGTGACAAA-3')(SEQ.ID.NO.30) and BB82. The second PCR reaction used oligos BB203 (5'-ACAAGATTTGGGCTCGGGCTGGGCAAG GTGGCGTAG-3')(SEQ.ID.NO.31) and BB91. The products from these PCR reactions were gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The approximate 1300 bp PCR product was gel-purified, digested with *Pfl*M1 and *Sac* II, and the ~ 400 bp *Pfl*MI/*Sac* II fragment gel-purified. This fragment was cloned into similarly cut pBBT174 that had been treated with calf intestinal phosphatase. A clone with the correct insert was identified by DNA sequencing and named pBBT299.

      A G-CSF-IgG4-Fc direct fusion was created by PCR using plasmid pBBT175 as the DNA template. One PCR reaction used oligos BB204 (5'- CACCTTGCCCAGCCCGAGTCCAAATATGGT CCCCCA-3')(SEQ.ID.NO.32) and BB82. The second PCR reaction used oligos BB205 (5'-ACCATATTTGGACTCGGGCTGGGCAAG GTGGCGTAG-3')(SEQ.ID.NO.33) and BB91. The products from these PCR reactions were gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The approximate 1300 bp PCR product was gel-purified, digested with and *Pfl*MIand *Sac* II, and the ~ 400 bp *Pfl*MI/*Sac* II fragment gel-purified. This fragment was cloned into similarly cut pBBT175 that had been treated with calf intestinal phosphatase. A clone with the correct insert was identified by DNA sequencing and named pBBT300.

      COS cells were transfected with pBBT299 and pBBT300 and the G-CSF-IgG1-Fc and G-CSF-IgG4-Fc direct fusion proteins purified as described in **Example 2**. The purified proteins, which consisted primarily (>90%) of disulfide-linked dimers, stimulated proliferation of NFS60 cells with an EC_{50} of 50-60 pg/ml (G-CSF-IgG1-Fc direct fusion) and 43-50 pg/ml (G-CSF-IgG4-Fc direct fusion). The bioassay was performed as described in **Example 3**.

2. GF-IgG Fusion Proteins Containing Two or Four Amino Acid Linkers

      To construct a di-peptide [ser-gly] linker, one can PCR the IgG1-Fc sequence with a 5' oligonucleotide that adds the 5' extension CGCTCCGGA to the hinge coding sequence. The TCCGGA hexanucleotide is a cleavage site for the restriction endonuclease *Bsp* EI and encodes amino acids ser-gly. This PCR fragment can be digested with *Bsp* EI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc. The unique *Bsp* EI site in each of these plasmids occurs at the first ser-gly in the linker [ser-gly-gly-ser-gly-gly-ser] so that the resulting recombinants will contain this 2 amino acid, ser-gly, linker. The sequence of the newly inserted ~ 250 bp *Bsp* EI - *Sac* II fragment can be verified.

      A similar procedure can be used to construct the 4 amino acid [ser-gly-gly-ser] linker. One can PCR the IgG1-Fc sequence with a 5' oligonucleotide that adds the 5' extension CGCGGATCC to the hinge coding sequence. The GGATCC hexanucleotide is a cleavage site for the restriction endonuclease *Bam* HI and encodes amino acids gly-ser. This PCR fragment can be digested with *Bam* HI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut pCDN3.1(+)::EPO-IgG1-Fc and

pCDNA3.1(+): G-CSF-IgG1-Fc. The unique *Bam* HI site in each of these plasmids occurs at the first gly-ser in the linker [ser-gly-gly-ser-gly-gly-ser] so the recombinants will contain the 4 amin acid (ser-gly-gly-ser) linker. The sequence of the inserted ~ 250 bp *Bam* HI – *Sac* II piece can be verified.

5 An IgG1-Fc domain that can be used to construct fusion proteins containing a two amino acid SerGly linker between the growth factor/cytokine domain and the IgG1-Fc domain was created by PCR. The PCR reaction used primers BB 194 (5'-CGCGAATTCCGGAGAGCCCAAATCTT GTGACAAA3')(SEQ.ID.NO.34) and BB 82. The DNA template was pBBT195. The PCR products were put through a PCR clean-up kit (Qiagen, Inc.) and digested with EcoRI and SacII. The restriction digest was put through a PCR clean-up kit and cloned into similarly cut pBC SK(+) (Stratagene, Inc.)

10 DNA that had been treated with calf intestinal phosphatase. The ligations were used to transform *E. coli* JM109. Transformants were selected on LB agar plates containing 50 µg/ml chloramphenicol. A clone with the correct sequence was identified by DNA sequencing and named pBBT242.

An IgG1-Fc domain that can be used to construct fusion proteins containing the four amino acid linker, Ser-Gly-Gly-Ser (SEQ.ID.NO.1), between the growth factor/cytokine domain and the

15 IgG1-Fc domain was created by PCR. The PCR reaction used primers BB 195 (5'-CGCGGATCC GAGCCCAAATCTTGTGACAAA-3')(SEQ.ID.NO.35) and BB 82. The DNA template was pBBT195. The PCR products were put through a PCR clean-up kit (Qiagen, Inc.) and digested with BamHI and Sac II. The restriction digest was put through a PCR clean-up kit and cloned into similarly cut pBC SK(+) DNA that had been treated with calf intestinal phosphatase. The ligations were used to

20 transform *E. coli* JM109. Transformants were selected on LB agar plates containing 50 µg/ml chloramphenicol. A clone with the correct sequence was identified by DNA sequencing and named pBBT243.

An IgG4-Fc domain that can be used to construct fusion proteins containing the two amino acid linker, SerGly, between the growth factor/cytokine domain and the IgG4-Fc domain was created

25 by PCR. The PCR reaction used primers BB 196 (5'-CGCGAATTCCGGAGAGTCCAAATATGG TCCCCCA-3')(SEQ.ID.NO.36) and BB 82. The DNA template was pBBT196. The PCR products were put through a PCR clean-up kit (Qiagen, Inc.) and digested with EcoRI and SacII. The restriction digest was put through a PCR clean-up kit and cloned into similarly cut pBC (SK+) DNA that had been treated with calf intestinal phosphatase. The ligations were used to transform E. coli JM109.

30 Transformants were selected on LB agar plates containing 50 µg/ml chloramphenicol. A clone with the correct sequence was identified by DNA sequencing and named pBBT244.

An IgG4-Fc domain that can be used to construct fusion proteins containing the four amino acid linker, ser-gly-gly-ser, between the growth factor/cytokine domain and the IgG4-Fc domain was created by PCR. The PCR reaction used primers BB 197 ((5'-CGCGGATCCGAGTCCAAATATGGT

35 CCCCCA-3')(SEQ.ID.NO.37) and BB 82. The DNA template was pBBT196. The PCR products were put through a PCR clean-up kit (Qiagen, Inc.) and digested with BamHI and Sac II. The restriction digest was put through a PCR clean-up kit and cloned into similarly cut pBC (SK+) DNA that had been treated with calf intestinal phosphatase. The ligati ns were used to transform E. coli JM109.

Transformants were selected on LB agar plates c ntaining 50 µg/ml chloramphenicol. A clone with the correct sequence was identified by DNA sequencing and named pBBT245.

The modified IgG domains described above were used to construct EPO-IgG and G-CSF-IgG fusion proteins containing four amino acid linkers. Plasmids pBBT174 and pBBT180 were digested

5   with BamHI and SacII and treated with calf intestinal phosphatase. The ~ 6 kb vector bands were gel-purified and ligated with the ~ 800 bp IgG insert from pBBT243, which had been gel-purified following digestion of pBBT243 DNA with BamHI and SacII. Similarly, plasmids pBBT175 and pBBT181 were digested with BamHI and SacII and treated with calf intestinal phosphatase. The ~ 6 kb vector bands were gel-purified and ligated with the ~ 800 bp IgG insert from pBBT245, which had

10   been gel-purified following digestion of pBBT245 DNA with BamHI and SacII. The ligations were used to transform E. coli DH5α and transformants selected on LB agar plates containing 100 µg/ml ampicillin. Clones containing the correct sequences were identified by restriction mapping and DNA sequencing. The EPO-IgG1-Fc and EPO-IgG4-Fc fusions containing the 4 amino acid linker were named pBBT279 and pBBT280, respectively. The G-CSF-IgG1-Fc and G-CSF-IgG4-Fc fusions

15   containing the 4 amino acid linker were named pBBT277 and pBBT278, respectively.

The modified IgG domains described above can be used to construct EPO-IgG and G-CSF-IgG fusion proteins containing two amino acid linkers. Plasmids pBBT174 and pBBT180 can be digested with BspEI and SacII and treated with calf intestinal phosphatase. The ~ 6 kb vector bands can be gel-purified and ligated with the ~ 800 bp IgG insert from pBBT242, which had been gel-

20   purified following digestion of pBBT242 DNA with BspEI and Sac II. Similarly, plasmids pBBT175 and pBBT181 can be digested with BspEI and Sac II and treated with calf intestinal phophatase. The ~ 6 kb vector bands can be gel-purified and ligated with the ~ 800 bp IgG insert from pBBT244, which has been gel-purified following digestion of pBBT244 DNA with Bsp EI and Sac II. The ligations can be used to transform E. coli DH5α and transformants selected on LB agar plates containing 100 µg/ml

25   ampicillin.

The recombinant fusion proteins can be used to transfect COS cells and be purified as described in Example 2. In vitro and in vivo bioactivities of these fusion proteins can be determined as described in the Examples 3 and 8.

30   Example 5

Methods to Improve Bioactivities of IgG-C_H Fusion Proteins

All of the IgG-C_H fusion proteins appeared to aggregate during purification and the specific activities of the fused growth factors were reduced ~ 2-3-fold as compared to the analogous IgG-Fc fusions. Aggregation may be due to hydrophobic interactions involving the CH1 domain that normally

35   interfaces with the light chain. Coexpression of Ig light chains with the GF-IgG-C_H fusions can prevent aggregation. We describe three ways to coexpress Ig light chains and Ig heavy chains below. The experiments described below use IgG-Fc, IgG-C_H, EPO and G-CSF as examples, however, similar procedures can be used for other GF or IgG domains, and domains for other IgG subtypes and domains from IgM, IgA,IgD and IgE antibodies. The DNA sequences of human kappa and lambda light chains

are known (Heiter et al., 1980). cDNA sequences encoding the human kappa and / or lambda light chain constant (CL) regions can be obtained by PCR amplification from the human leukocyte single-stranded cDNA (Clontech) or human genomic DNA (Clontech). The DNA sequences of the cloned CL domains can be confirmed prior to use in the experiments described below. The modified fusion

5   proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in Examples 2 and 3.

1. Co-expression of the light chain constant region. A Kozak sequence (Kozak, 1991) and a secretion signal can be added to the 5' end of the light chain constant region to enhance translational

10   initiation and direct the secretion of the light chain constant region. A translational stop codon can be added to the 3' end of the sequence. Appropriate cloning sites can be added to the 5' and 3' ends to allow cloning into the mammalian cell expression vector pREP4 (Invitrogen) under control of the RSV promoter and preceding the SV40 derived polyA addition site. This construct can be used to cotransfect COS cells along with pCDNA3.1(+) derivatives that express, for example, EPO-IgG-C_H and G-CSF-

15   IgG-C_H. Alternatively, both light and heavy chains can be expressed from a single plasmid construct. In this case, the light chain sequence and the flanking promoter and polyA sites from pREP4 can be excised with appropriate restriction enzymes and cloned into pCDNA3.1(+). The EPO-IgG-C_H and G-CSF-IgG-C_H coding sequences could then cloned into the pCDNA3.1(+) polylinker under control of the CMV promoter.

20   DNA encoding the constant region of the human Kappa light chain (IgKC) was amplified from human genomic DNA as described below in Section 2 of this Example. This sequence was modified by PCR to create a construct suitable for expression of the light chain constant region. A Kozak sequence (Kozak, 1991) and the human Growth Hormone secretion signal were added to the 5' end of the light chain constant region. These modifications were made via two sequential PCR

25   reactions. In the first the cloned and sequenced pCDNA3.1(+)::fusIgKC DNA was used as template. The primers were BB169 (5>GCTTTTGGCCTGCTCTGCCTGCCC TGGCTTCAAGAGGGCAGTGCCACTGTGGCTGCACCATCT>3)(SEQ.ID.NO.38) and BB150 BB150 (5> CGCTCTAGACTA ACACTCTCCCCTGTTGAA >3)(SEQ.ID.NO.39). Forward primer BB169 anneals to the 5' end of the light chain constant region and adds sequence encoding 15 amino

30   acids of the hGH leader sequence. Reverse primer BB150 anneals to the 3' end of the coding sequence for the constant region of the human Kappa light chain and includes a translational stop codon followed by an *Xba* 1 site. The product of this PCR reaction was gel-purified and used as template in a second PCR reaction with primers BB168 (5>CGCAAGCTTGCCACCATGGCTACAGGC TCCCGGACGTCCCTGCTCCTGGCTTTTGGCCTGCTCTGC>3)(SEQ.ID.NO.40) and BB150.

35   Forward primer BB168 adds the remainder of the hGH leader sequence as well as a Kozak sequence and contains a *Hin* dIII site for cloning purposes. Reverse primer BB150 is described above. The product of this PCR reacti n was digested with *Hin* dIII and *Xba* 1 and cloned into vector pCDNA3.1(+) that had been digested with *Hin* dIII and *Xba* 1 and treated with alkaline phosphatase. The DNA sequence was verified for one such clone.

2. Co-expression of GF–light chain constant region fusion proteins. An alternative mode of light chain expression would be to modify the 5' end to add a portion of a flexible peptide linker sequence fused to the amino-terminus of the CL coding sequence and add a translational stop codon to the 3' end of the sequence. Appropriate cloning sites can be added as well to the 5' and 3' ends to allow cloning as an in frame fusion to the EPO and G-CSF genes cloned in the plasmids pCDNA3.1(+)::EPOfus and pCDNA3.1(+)::G-CSFfus. This plasmid can be cotransfected into COS cells with plasmids that express, for example, EPO-IgG1-C_H and G-CSF-IgG1-C_H. In this instance both heavy and light chains will contain growth factor fusions. The light and heavy chains also could be expressed from a single pCDNA3.1 (+) construct as described above. . EPO and G-CSF also can be expressed as direct fusions with Ig-C_L using procedures similar to those described elsewhere in the Examples.

DNA encoding the constant region of the human Kappa light chain (IgKC) was amplified from human genomic DNA (CLONTECH, Inc., Palo alto, CA) by PCR using primers BB149 (5>CGCGGATCCGGTGGCTCAACTGTGGCTGCACCATCTGT >3)(SEQ.ID.NO.41) and BB150 (5> CGCTCTAGACTA ACACTCTCCCCTGTTGAA >3)(SEQ.ID.NO.42). Forward primer BB149 anneals to the 5' end of the coding sequence for the constant region of the human Kappa light chain and includes a portion of the peptide linker (gly-ser-gly-gly-ser)(SEQ.ID.NO.2) containing a *Bam* HI site at the 5' end. Reverse primer BB150 anneals to the 3' end of the coding sequence for the constant region of the human Kappa light chain and includes a translational stop codon followed by an *Xba* I site. The resulting ~ 330 bp PCR product was digested with *Bam* HI and *Xba* I, gel purified and cloned into vector pCDNA3.1(+) that had been digested with *Bam* HI and *Xba* I, alkaline phosphtase treated, and gel purified. One clone was sequenced and found to match the published human IgKC genomic DNA sequence (Hieter et al., 1980). The ~330 *Bam* HI - *Xba* I fragment was subsequently excised from this plasmid, termed pCDNA3.1(+)::fusIgKC, and cloned into pBBT190, pBBT191, and PBBT192 (described in Example 10 below) resulting in fusion of the IgKC domain to IFN-α, IFN-β, and IFN-γ respectively. Similar procedures can be used to construct other growth factor/cytokine-IgKC fusion proteins.

3. Light chain-heavy chain fusions. A third mode of "co-expression" would be to modify the 5' and 3' ends of the CL coding sequence to incorporate portions of a flexible peptide linker at both ends. By also incorporating appropriate cloning sites (e.g. *Bsp* EI and *Bam* HI) such a construct can be inserted into the Bsp EI and *Bam* HI sites within the flexible peptide linkers of the EPO-IgG-C_H and G-CSF-IgG-C_H fusions in pCDNA3.1(+). The resulting constructs would encode, for example, single polypeptide [EPO]-[CL]-[IgG-C_H] and [G-CSF]-[CL]-[IgG-C_H] fusions. The fusion of the carboxy-terminus of the light chain constant region to the amino-terminus of the heavy chain CH1 domain would be analogous to single chain Fv polypeptides. Flexible peptide linkers of the (ser-gly-gly) motif on the order 14 to 20 residues in length have been used to fuse the carboxy-terminus of the light chain variable region to the amino-terminus of the heavy chain variable domain (Stewart et al., 1995) and c uld be used to join the CL domain t the IgG-C_H domain.

Example 6

GF-IgG Fusion Proteins with Reduced Complement Binding
and Fc Receptor Binding Properties

Certain GF-IgG1 fusion proteins may be toxic or lack efficacy in the animal models due to activation of complement or immune processes related to Fc receptor binding. For this reason, GF-IgG4 fusion proteins may be preferred because IgG4 is less efficient at complement activation and Fc receptor binding than is IgG1 (Roit et al., 1989). The EPO- and G-CSF-IgG4-Fc fusion proteins are as potent or nearly as potent as the IgG1-Fc fusion proteins in *in vitro* bioassays. Alternatively, one can perform *in vitro* mutagenesis experiments, as detailed below, to change specific amino acids in the IgG domains known to be responsible for complement activation and Fc receptor binding. The modified fusion proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in Examples 2 and 3.

A. Complement Binding. Amino acids in IgGs that play a role in complement activation have been localized to the IgG CH2 domain. Specifically, amino acids Glu318, Lys320, Lys322, Ala330 and Pro331 in human IgG1 have been implicated as contributing to complement activation (Isaacs et al., 1998). Substitution of Glu318, Lys320 and Lys322 in IgG1 with alanine residues results in IgG proteins possessing reduced ability to activate complement (Isaacs et al., 1998). The amino acid sequence of IgG4 is identical to IgG1 in this region, yet IgG4 does not activate complement. As an alternative to using IgG4, one can change Glu318, Lys320 and Lys 322 (alone or in combination) of IgGs that have these residues to alanine residues or other amino acids that reduce complement activiation using the PCR-based mutagenesis strategies described in Example 1.

B. Fc Receptor Binding. Human IgG subclasses differ in their ability to bind Fc receptors and stimulate antibody-dependent cell-mediated cytotoxity (ADCC). IgG1, IgG3 and IgG4 are best at stimulating ADCC, whereas IgG2 has significantly reduced ability to stimulate ADCC (Roit et al., 1989). ADCC occurs through a mechanism that involves binding of the antibody to Fc receptors on immune cells. Amino acids responsible for Fc receptor have been localized to the CH2 domain of the IgG molecule. Specifically, amino acids 233-235 have been implicated in Fc receptor binding. Human IgG1 has the amino acid sequence GluLeuLeu in this region, whereas IgG2, which does not bind Fc receptors, has the sequence ProAlaVal. IgG4 has the sequence GluPheLeu in this region and is 10-fold less efficient at binding Fc receptors than IgG1. Substitution of the IgG2 sequence ProAlaVal for GluLeuLeu at positions 233-235 in IgG1or IgG4 results in IgG1 and IgG4 antibodies with significantly reduced capacity for Fc receptor binding and ADCC (Isaacs et al., 1998). One can introduce some or all of these amino acid changes into the GF-IgG fusion protein constructs using the PCR-based mutagenesis strategy described in Example 1. Alternatively one can construct modified GF-IgG fusion proteins in which glycosylation of asparagine 297 in the IgG1 CH2 domain (or the equivalent asparagine residue in the other IgG subclasses) is prevented. Aglycosylated IgG1 antibodies display

significantly reduced binding to Fc receptors and ability to lyse target cells as compared to glycosylated IgG1 antibodies (Isaacs et al., 1998). One can construct aglycosylated versions of the GF-IgG fusion proteins by changing asparagine-297 to glutamine or another amino acid, or by changing threonine-299, which is part of the glycosylation recognition sequence (Asparagine-X-Serine/Threonine), to alanine or to an amino acid other than serine. The amino acid in the X position of the glycosylation recognition sequence, i.e, amino acid 298, also could be changed to proline to prevent glycosylation of asparagine 297 in the IgG CH2 domain.

Example 7
Pharmacokinetic Experiments with GF-IgG Fusion Proteins

Pharmacokinetic experiments can be performed to demonstrate that the GF-IgG fusion proteins have longer circulating half-lives than the corresponding non-fused proteins. Both intravenous and subcutaneous pharmacokinetic data can be obtained. Terminal pharmacokinetic parameters can be calculated from the intravenous delivery data.

For the intravenous delivery studies, rats (~350g) can receive an intravenous bolus injection (0.1 mg/kg) of the IgG1-Fc fusion protein (EPO or G-CSF) or the corresponding non-fused protein (EPO or G-CSF) and circulating levels of the proteins measured over the course of 144 h. Three rats can be used for each protein sample. Blood samples can be drawn at 0, 0.08, 0.5, 1.5, 4, 8, 12, 24, 48, 72, 96, 120, and 144 h following intravenous administration. Serum levels of the test proteins can be quantitated using commercially available EPO and G-CSF ELISA kits (R & D Systems). Serial dilutions of each blood sample can be analyzed initially in the *in vitro* bioassays to identify dilutions that will fall within the linear range of the ELISAs. (0.025 to 1.6 ng/ml for EPO and 0.04 to 2.5 ng/ml for G-CSF). Titration experiments can be performed to determine the relative sensitivities of the ELISAs for detecting the IgG1-Fc fusion proteins and the corresponding non-fused proteins. The subcutaneous delivery studies can follow the same protocol as the intravenous studies except for the route of delivery. Serum levels of the test proteins can be quantitated by ELISA as described above.

Example 8
Animal Efficacy Models

In vivo efficacy of the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins can be demonstrated in normal rats and mice. These studies can use a variety of doses and dosing schedules to identify the proper doses and dosing schedules. Efficacy of the EPO-IgG and G-CSF-IgG fusion proteins also can be demonstrated in appropriate disease models – anemia for EPO-IgG1-Fc and neutropenia for G-CSF-IgG1-Fc. The pharmacokinetic experiments will provide guidance in deciding dosing schedules for the IgG1 fusion proteins to be used for the animal studies. From published results with other IgG-Fc fusion proteins (Richter et al., 1999; Zeng et al., 1995) the EPO-IgG and G-CSF-IgG fusion proteins can be effective when administered every other day or every third day and possibly less ften, e.g. a single injection. The d sing schedules may have to be modified depending upon the results of the pharmacokinetic studies and initial animal efficacy results. The dose of protein administered per

injection to the rodents also may have to be modified based upon the results of the pharmacokinetic experiments and initial animal efficacy results

A. EPO Animal Efficacy Models – N rmal Rats

5   **1. Single injection:**

Groups of three male Sprague Dawley rats, weighing ~ 320g each, received a single intravenous injection (lateral tail vein) of wild type recombinant EPO containing the amino acid sequence ser-gly-gly-ser-gly-gly-ser-asp-tyr-lys-asp-asp-asp-asp-lys (SEQ.ID.NO.43) at its carboxy end (rEPO-FLAG), or EPO-IgG1-Fc at a dose of 100 μg/kg. The rEPO-FLAG was expressed by

10   transfection of COS-1 cells and purified by affinity chromatography using an anti-FLAG monoclonal antibody. Protein concentrations of rEPO-FLAG and EPO-IgG1-Fc were determined using a Bradford dye binding assay and bovine serum albumin as the standard. At selected time points blood samples (0.3 to 0.4 ml) were drawn from the rats into EDTA anti-coagulant tubes. Aliquots of the blood samples were analyzed for a complete blood cell (CBC) count. The remainder of the blood sample

15   was centrifuged and the plasma frozen at -80^0C. Blood samples were drawn at 0.25 , 2, 4, 10, 24, 48, 72, 96, 120, 144, 168 and 192 h post-injection. A 0 h baseline sample was obtained ~ 24 h prior to injection of the test compounds. Table 4 shows the mean blood hematocrits (+/- SE) for the different test groups at 0 hour and at 192h post-injection. The group receiving EPO-IgG1-Fc showed a significant increase in hematocrit levels at 192 h compared to 0 hour ($p < 0.05$). The group receiving

20   rEPO-FLAG did not show a significant increase in hematocrit levels between the 0 and 192 hour time points.

Table 4

Group	Hematocrit (%)	
	0 hour	192 hour
rEPO-FLAG	42.9 +/- 0.85	44.4 +/- 1.007
EPO-IgG1-Fc	44.7 +/- 0.296	[*] 48.0 +/- 0.95

[*] $p < 0.05$ versus 0 hour time point.

25   Similar studies can be performed using lower or higher doses (0.001 to 10 mg/kg) of the proteins. Similar studies also can be performed using the subcutaneous route of administration of the proteins.

2. Daily, Every Other Day or Every Third Day dosing Schedules

Sprague-Dawley rats (~200g) can be purchased from a commercial supplier such as Charles River

30   (Wilmington, MA). Previous studies have shown that administration of 100 – 1000 IU/kg (approximately 800 ng – 8 μg/kg) of rEPO once per day (160 ng – 1.6 μg SID / 200 g rat) by subcutaneous injection gives a significant increase in hematocrit and erythropoiesis in rodents (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998; Sytkowski et al., 1998). Groups of 5 rats can receive subcutaneous injections of rEPO, rEPO-FLAG, EPO-IgG1-Fc or placebo (vehicle

35   solution) at specified intervals (daily, every other day or every third day) for five to nine days. A dose equivalent to a molar ratio of EPO (400 ng – 4μg/200g rat of the EPO-IgG1-Fc fusion protein) can be

tested. Higher r lower doses also can be tested. A wide range of EPO-IgG1-Fc doses (over 500-fold
variation) can be tested in these initial experiments to increase the likelihood that one of the doses will
be effective. It is possible that administration of too much EPO-IgG1-Fc will impede erythropoiesis
due to toxicity. Control rats can receive vehicle solution only. Additional control groups can receive
5   daily injections of rEPO (160 ng-1.6 μg/200g rat for 5-9 days) and 160 ng –1.6 μg rEPO using the
same dosing regimen as EPO-IgG1-Fc. One to two days after the final injection the animals can be
sacrificed and blood samples collected for hematocrit and complete blood cell count (CBC) analysis.
Hematopoietic tissues (liver and spleen) can be collected, weighed and fixed in formalin for
histopathologic analyses to look for evidence of increased erythropoiesis. Bone marrow can be
10   removed from various long bones and the sternum for unit particle preps and histopathologic analysis
to look for evidence of increased erythropoiesis. Comparisons between groups can be made using a
Students T test for single comparisons and one-way analysis of variance for multiple comparisons. P<
0.05 can be considered significant.

       Daily injections of rEPO can stimulate increases in hematocrit and erythropoiesis in the rats,
15   whereas less frequent administration of the same dose of rEPO can not, or do so to a lesser extent.
Dose-dependent increases in these parameters can be observed in the EPO-IgG-Fc-treated animals.
Greater increases in these parameters may be observed in the EPO-IgG1-Fc-treated animals than in
animals treated with EPO using the less frequent dosing schedules. Significantly less EPO-IgG1-Fc
may be required to achieve the same increases in these parameters obtained with daily injections of
20   EPO.

       Additional experiments with less frequent dosing, e.g., a single injection, could be performed.

B. EPO Efficacy Experiment - Anemic Rat Model

       Cisplatin-induced anemia is a well-characterized rodent model of chemotherapy-induced
25   anemia and has direct relevance to the human clinical setting. rEPO reverses the anemia in this model
when administered at daily doses of 100 –1,000 Units/kg (Matsumoto et al., 1990; Vaziri et al., 1994;
Baldwin et al., 1998). EPO-IgG-Fc also will be effective at reversing anemia in this model using once
per day, every other day or every third day dosing schedules. The dosing schedule for EPO-IgG-Fc to
be used in this experiment could be the one that worked best in the normal rat experiments. Sprague-
30   Dawley rats (~200g) can be treated on day 0 with an intraperitoneal injection of Cisplatin (3.5mg/kg) to
induce anemia and randomized to various treatment groups. Rats can receive daily, every other day or
every third day injections of EPO-IgG-Fc, rEPO or saline for up to 9 days at a dose of 2μg – 20μg/kg.
One control group of rats can receive daily subcutaneous injections of rEPO (100-1,000 Units/kg).
Another control group can not receive the initial Cisplatin injection but can receive injections of saline
35   using the same dosing schedule as the other test proteins. On day 9 the rats can be sacrificed and blood
and tissue samples obtained for comprehensive CBC and histopathology analyses. Ten to one hundred
f ld higher or lower doses of the EPO-IgG1-Fc fusion proteins also c uld be tested.

C. G-CSF Animal Efficacy Models

In vivo efficacy of the G-CSF-IgG fusion proteins (C17 or C17S versions) can be measured in normal or neutropenic rodents such as mice or rats by demonstrating that the proteins stimulate increases in circulating neutrophil levels and granulopoiesis compared to vehicle-treated animals. G-CSF stimulates neutrophil levels in normal and neutropenic rodents at a dose of 100 µg/kg (Kubota et al., 1990; Kang et al., 1995). Mice or rats can be used for these experiments. One can extrapolate pharmacokinetic data from the rat to the mouse because protein clearance is proportional to body weight (Mahmood, 1998). One can demonstrate efficacy of G-CSF-IgG1-Fc in normal animals using daily, every other day or every third day dosing schedules. Efficacy also can be demonstrated following a single injection. Effectiveness of G-CSF-IgG1-Fc in a mouse neutropenia model also can be demonstrated using daily, every other day or every third day dosing schedules.

C. 1. Efficacy in Normal Rats Following a Single Injection

Groups of three male Sprague Dawley rats, weighing ~ 320g each, received a single intravenous injection (lateral tail vein) of wild type recombinant G-CSF (prepared by Bolder BioTechnology), Neupogen® (a recombinant G-CSF sold by Amgen, Inc.) or G-CSF-IgG1-Fc, each at a dose of 100 µg/kg. Protein concentrations were determined using a Bradford dye binding assay using bovine serum albumin as the standard. At selected time points blood samples (0.3 to 0.4 ml) were drawn from the rats into EDTA anti-coagulant tubes. Aliquots of the blood samples were analyzed for a complete blood cell (CBC) count. The remainder of the blood sample was centrifuged and the plasma frozen at -80^{0}C. Blood samples were drawn at 0.25, 1.5, 4, 8, 12, 16, 24, 48, 72, 96, 120 and 144h post-injection. A 0 h baseline sample was obtained ~ 24 h prior to injection of the test compounds. Tables 5, 6 and 7 show the mean blood neutrophil, total white blood cell counts and blood monocyte counts for the different test groups over time. All three test compounds stimulated an increase in peripheral white blood cells and neutrophils over baseline values. White blood cell and neutrophil counts for the test groups receiving wild type recombinant G-CSF and Neupogen® peaked ~ 24 h post-injection and returned to baseline values by ~ 48 h. In contrast, white blood cell and neutrophil counts for the rats receiving G-CSF-IgG1-Fc peaked ~48 h post-injection and did not return to baseline values until ~ 96 h post-injection. Peak white blood cell and neutrophil levels observed in the rats receiving G-CSF-IgG1 were significantly higher than for the groups receiving wild type recombinant G-CSF or Neupogen® (p<0.05). The data indicate that G-CSF-IgG1 is capable of stimulating an increase in circulating neutrophil and white blood cells, and that the absolute increase in peripheral white blood cell counts and neutrophils is greater and longer lasting than that seen with recombinant wild type G-CSF or Neupogen®, even when using a lower molar dose of the fusion protein. G-CSF-IgG1-Fc also stimulated a significant increase in blood monocyte levels over time (Table 7). Similar experiments can be performed to demonstrate efficacy of other G-CSF-IgG and G-CSF (C17S)-IgG fusion proteins (Fc or C_H versions). Similar studies also can be performed using the subcutaneous route for administration of the proteins.

Table 5

Effects of G-CSF, Neupogen® and G-CSF-IgG1-Fc on Neutrophil Blood Cell Counts Following Single Intravenous Administration of the Proteins (100 µg/kg)

Time (Hr)	Neutrophils Mean +/- SE (cells/µl blood)		
	G-CSF[a]	Neupogen	G-CSF-IgG1-Fc
0	1,147 +/- 167	1,906 +/- 564	2,951 +/- 342
4	6,752 +/- 923	4,504 +/- 549	[b] 4,484 +/- 328
8	8,437 +/- 546	5,525 +/- 894	[b] 7,309 +/- 890
12	10,744 +/- 549	11,891 +/- 1,545	[b] 9,796 +/- 1,649
24	11,035 +/- 788	11,148 +/- 977	[b] 13,655 +/- 2,367
48	2,355 +/- 218	2,610 +/- 245	[b,c] 14,554 +/- 1,683
72	2,113 +/- 438	3,077 +/- 590	[b,c] 6,235 +/- 797
96	2,086 +/- 496	2,675 +/- 673	3,292 +/- 309
120	2,179 +/- 373	2,063 +/- 469	3,115 +/- 342

[a] Wild type G-CSF prepared by Bolder BioTechnology, Inc.
[b] $p < 0.05$ versus 0 hour neutrophil levels
[c] $p < 0.05$ versus G-CSF and Neupogen at same time point

Table 6

Effects of G-CSF, Neupogen® and G-CSF-IgG1-Fc on White Blood Cell Counts Following Single Intravenous Administration of the Proteins (100 µg/kg)

Time (Hr)	White Blood Cells Mean +/- SE (cells/µl blood)		
	G-CSF[a]	Neupogen	G-CSF-IgG1-Fc
0	11,100 +/- 252	11,100 +/- 829	14,000 +/- 669
4	16,000 +/- 1,059	13,600 +/- 570	13,600 +/- 186
8	15,200 +/- 371	14,900 +/- 260	15,300 +/- 1,670
12	18,400 +/- 240	20,100 +/- 674	19,400 +/- 2,058
24	23,900 +/- 1,110	25,500 +/- 1,734	[b] 29,300 +/- 2,894
48	14,700 +/- 426	15,300 +/- 1,715	[b,c] 33,100 +/- 2,099
72	15,300 +/- 426	14,800 +/- 764	[b,c] 21,200 +/- 2,228
96	14,200 +/- 1,000	14,700 +/- 689	15,400 +/- 133
120	11,000 +/- 2,651	11,300 +/- 1,477	12,900 +/- 2,052

[a] Wild type G-CSF prepared by Bolder BioTechnology, Inc.
[b] $p < 0.05$ versus 0 hour white blood cell levels
[c] $p < 0.05$ versus G-CSF and Neupogen at same time point

Table 7

Effects of G-CSF, Neupogen® and G-CSF-IgG1-Fc on Blood Monocyte Cell Counts Following

Single Intravenous Administration of the Proteins (100 µg/kg)

Time (Hr)	Monocytes Mean +/- SE (cells/µl blood)		
	G-CSF[a]	Neupogen	G-CSF-IgG1-Fc
0	622 +/- 255	675 +/- 163	804 +/- 208
4	1,837 +/- 60	1,320 +/- 215	1,364 +/- 296
8	1,519 +/- 168	1,044 +/- 79	1,025 +/- 234
12	1,166 +/- 51	1,482 +/- 315	999 +/- 273
24	1,283 +/- 140	1,092 +/- 5	1,463 +/- 145
48	638 +/- 68	825 +/- 147	3,167 +/- 111
72	820 +/- 293	1,079 +/- 80	2,416 +/- 323
96	893 +/- 363	1,175 +/- 147	1,118 +/- 57
120	786 +/- 56	557 +/- 64	1,208 +/- 294

[a] Wild type G-CSF prepared by Bolder BioTechnology, Inc.

Plasma G-CSF and G-CSF-IgG protein levels can be quantitated using commercially available G-CSF ELISA kits (R & D Systems, Inc.). Titration experiments can be performed to determine the relative sensitivity of the ELISA for detecting wild type G-CSF and G-CSF-IgG fusion proteins. Similar studies can be performed using the subcutaneous route of administration of the proteins.

C. 2. G-CSF-IgG Fusion Protein Efficacy in Normal Mice

For demonstrating efficacy in normal mice, groups of 5 mice (weighing ~ 20 g each) can receive subcutaneous injections of G-CSF, Neupogen, G-CSF-IgG fusion proteins or placebo (vehicle solution) at specified intervals for up to five days. Normal mice such as ICR mice can be purchased from a commercial vendor such as Jackson Laboratories, Charles River or Harland Sprague Dawley. On day 6 the animals can be sacrificed and blood samples collected for complete blood cell count (CBC) analysis. Hematopoietic tissues (liver and spleen) can be collected, weighed and fixed in formalin for histopathologic analyses to look for evidence of increased granulopoiesis. Bone marrow can be removed from various long bones and the sternum for unit particle preps and histopathologic analysis to look for evidence of increased granulopoiesis. Comparisons between groups can be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. P< 0.05 can be considered significant. The G-CSF-IgG fusion proteins can stimulate greater increases in circulating neutrophil levels and granulopoiesis in the mice compared to the vehicle-treated mice. Efficacy of the G-CSF-IgG fusion proteins can be tested when administered once on day 1, once per day on days 1-5, every other day on days 1, 3 and 5, or every third day on days 1 and 4. In initial experiments, different groups f mice (weighing ~20g each) can receive subcutaneous injections f 0.008 to 20 µg per injection of the G-CSF-IgG fusion proteins. Control mice can receive vehicle solution only. Additional control groups can receive wild type G-CSF or Neupogen (2 µg/

every day for 5 days) and 2μg wild type G-CSF using the same dosing regimen as the G-CSF-IgG
fusion proteins.

Daily injections of rG-CSF can stimulate increases in circulating neutrophils and
granulopoieis in the mice, whereas less frequent administration of the same dose of rG-CSF can not, or

5   can do so to a lesser extent. Dose-dependent increases in these parameters can be observed in the G-
CSF-IgG1-Fc-treated animals. Greater increases in these parameters may be observed in the G-CSF-
IgG1-Fc-treated animals than in animals treated with rG-CSF using the less frequent dosing schedules.
Significantly less G-CSF-IgG1-Fc may give the same increases in these parameters obtained with daily
injections of rG-CSF.

10

3. G-CSF-IgG Fusion Protein Efficacy in Neutropenic Mice: Efficacy of G-CSF-IgG1-Fc also can
be demonstrated in neutropenic animals. Neutropenia can be induced by treatment with
cyclophosphamide (CPA; 100 mg/kg), which is a commonly used chemotherapeutic agent that is
myelosuppressive and relevant to the human clinical setting. G-CSF accelerates recovery of normal

15   neutrophil levels in cyclophosphamide-treated animals (Kubota et al., 1990; Kang et al., 1995). Mice
(~20g) can receive an intraperitoneal injection of cyclophosphamide on day 0 to induce neutropenia.
The animals can be divided into different groups of 5 animals each, which will receive subcutaneous
injections of G-CSF, G-CSF-IgG1-Fc or placebo for up to five days post-CPA treatment. One control
group can not receive cyclophosphamide but can receive placebo injections. The exact dosing schedule

20   to be used can be determined by the results of the pharmacokinetic experiments and normal mouse
efficacy studies described above. Experiments can be performed using every day, every other day or
every third day dosing schedules over the course of five days. Doses of G-CSF-IgG1-Fc to be tested
can be 0.008 –20 μg/injection. On day six the animals can be sacrificed and blood and tissue samples
analyzed as described above. Alternatively, five mice per group can be sacrificed on days 0-10 and

25   blood and tissue samples analyzed as described for the normal mouse experiments above. The G-CSF-
IgG fusion proteins can stimulate an accelerated increase in circulating neutrophil levels and
granulopoiesis in the mice compared to the vehicle-injected, CPA-injected control group.

C.   GH-IgG Fusion Protein Efficacy

30   In vivo efficacy of the GH-IgG fusion proteins can be tested in hypophysectomized (HYPOX)
rats. This is a well-characterized model of GH deficiency (Cox et al., 1994; Clark et al., 1996). GH
stimulates body weight gain and bone and cartilage growth in HYPOX rats (Cox et al., 1994; Clark et
al., 1996). Hypophysectomized Sprague-Dawley rats can be purchased from a commercial supplier
such as Charles River (Wilmington, MA). Typically, rats are hypophysectomized between 40 and 50

35   days of age and weigh approximately 100-120g. Groups of 5-8 rats can receive subcutaneous
injections of rhGH, GH-IgG or placebo (vehicle solution) at specified intervals and weight gain
measured daily over a 10 day period. Rats can be weighed daily at the same time per day to minimize
possible variables associated with feeding. In addition to overall weight gain, bone growth (tibial
epiphysis width) can be measured. At time of sacrifice, the right and left proximal tibial epiphyses can

be removed and fixed in formalin. The fixed tibias can be split at the proximal end in a saggital plane, stained with silver nitrate and exposed to a strong light (Greenspan et al., 1949). The width of the cartilaginous epiphyseal plate can be measured using a stereomicroscope equipped with a micrometer eyepiece. Ten measurements can be made for each epiphysis and the means +/- SEM for the combined

5   values for the left and right tibias can be calculated. Comparisons between groups can be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. $P<$ 0.05 can be considered significant.

Efficacy of the GH-IgG fusion proteins can be tested by administering the proteins to the rats daily, every other day, every third day, every fourth day or following a single injection. Five µg of

10 hGH administered twice a day (10µg/day) by subcutaneous injection gives a strong growth response in the HYPOX rat model (Cox et al., 1994; Clark et al., 1996). In initial experiments different groups of rats can receive subcutaneous injections of various doses of GH-IgG ranging from 0.2 to 200 µg/injection/rat. Control rats can receive vehicle solution only. Additional control groups can receive rhGH (5 µg BID) and 10 µg hGH using the same dosing regimen as the GH-IgG fusion proteins.

15 Administration of the GH-IgG fusion proteins to the HYPOX rats can result in an increase in body weight gain and tibial epyphysis width growth compared to the vehicle-treated group.

Efficacy of the GH-IgG fusion proteins also can be tested in rodent models of cachexia. Dexamethasone (DEX) can be administered to the rats to induce weight loss. Groups of normal Sprague-Dawley rats (200 – 225g) can receive daily subcutaneous injections of dexamethasone (200

20 µg/rat; approximately 1 mg/kg). This amount of dexamethasone can induce a loss of approximately 5-6g over an 8d period. Vehicle or varying doses of the GH-IgG can be administered to the rats once, daily, every other day, every third day or every fourth day in different experiments. Different groups of rats can receive subcutaneous injections of 0.2 to 400 µg of the GH-IgG fusion proteins/injection/rat. Additional controls can include a group of rats that will receive no DEX or injections, a group of rats

25 that receives DEX and rhGH (5 µg BID or 10 µg SID) and a group of rats that receives DEX and rhGH (10 µg daily, every other day, every third day, or every fourth day, depending upon the experiment, i.e., frequency that the GH-IgG fusion proteins are administered). Animals can be weighed daily. Food and water consumption can be monitored daily. At time of sacrifice, internal organs can be weighed. Statistical analyses can be performed as described for the HYPOX rat studies. Animals treated with

30 the GH-IgG fusion proteins can lose less weight than the vehicle-treated animals.

Example 9
Further Purification of IgG fusion Protein Dimers

The final purification scheme for the growth factor/cytokine-IgG fusion proteins could include

35 additional column chromatography steps in addition to affinity chromatography to remove protein contaminants and aggregates and to prepare purer preparations of fusion protein tetramers, dimers and monomers. Purer preparations of the Ig fusion proteins can improve their specific activities in in vitro bioassays. For use as human therapeutics it will be preferable to obtain preparations of growth factor/cytokine-IgG mon mers substantially free from fusion protein dimers, and preparations of IgG

fusion protein dimers substantially free from fusion protein monomers. Growth factor/cytokine-IgG dimers can be separated from IgG fusion protein monomers using a variety of column chr matography procedures known to those with skill in the art. Chromatography procedures also can be used to purify Ig fusion proteins from aggregates and other contaminating proteins. Examples of such

5   chromatography procedures include ion-exchange, size exclusion, hydrophobic interaction, reversed phase, metal chelation, affinity columns, lectin affinity, hydroxy apatite and immobilized dye affinity chromatography. Other useful separation procedures known to those skilled in the art include salt precipitation, solvent precipitation/extraction and polyethylene glycol precipitation. Endotoxin levels in the purified proteins can be tested using commercially available kits to ensure that they are not

10   pyrogenic.

The EPO-IgG4-Fc direct fusion described in Example 4 (encoded by pBBT273) was purified by Protein A affinity chromatography as described in Example 2. The purified protein was further purified by size exclusion chromatography using a Superdex 200 HR 10/30 column. The buffer was 20 mM NaPO4, pH 7.0, 150 mM NaCl. The column volume was 24 ml. The column was eluted with 1.5

15   column volumes of buffer at a flow rate of 0.5 ml/minute. Fractions of 0.5 ml were collected. A single major protein peak was observed, which eluted with an apparent molecular weight of 204 kDa. The column was calibrated using protein standards purchased from Sigma Chemical Company (catalogue # MW-GF-200). SDS-PAGE analysis under reducing and non-reducing conditions indicated that the major protein peak consisted primarily of disulfide-linked EPO-IgG4-Fc dimers (at least 90%, and

20   probably >95% by non-reducing SDS-PAGE). Large molecular weight contaminants (apparent molecular weights > 200 kDa by non-reducing SDS-PAGE) and low molecular weight contaminants (apparent molecular weights < 50 kDa by non-reducing SDS-PAGE) were largely removed by this column step.

Similar experiments were performed with the EPO-IgG1-Fc fusion protein encoded by

25   pBBT180, the G-CSF-IgG1-Fc fusion protein encoded by pBBT174 and the G-CSF-IgG4-Fc fusion protein encoded by pBBT175. These proteins were purified by Protein A affinity chromatography as described in Example 2 and then further purified by size-exclusion chromatography as described above. The EPO-IgG1-Fc fusion protein eluted from the sizing column with an apparent molecular weight of 219 kDa and consisted primarily of EPO-IgG1-Fc dimers (>95% by non-reducing SDS-PAGE). The G-

30   CSF-IgG1-Fc fusion protein eluted with an apparent molecular weight of 148 kDa and consisted primarily of G-CSF-IgG1-Fc dimers (>95% by non-reducing SDS-PAGE). The G-CSF-IgG4-Fc fusion protein eluted with an apparent molecular weight of 135 kDa and consisted primarily of G-CSF-IgG4-Fc dimers (at least 90%, and probably >95% by non-reducing SDS-PAGE). Large molecular weight contaminants (apparent molecular weights > 200 kDa by non-reducing SDS-PAGE) and low molecular

35   weight contaminants (apparent molecular weights < 50 kDa by non-reducing SDS-PAGE) were largely removed by this column step.

Example 10

The procedures described in the preceding examples can, with minor modifications, be used to created IgG fusions with other proteins. Examples of other IgG fusion proteins that would find therapeutic uses in humans include IgG fusions of other members of the GH supergene family, including interferons alpha, beta and gamma, IL-11, TPO, GM-CSF, Stem cell factor and flt3 ligand. DNAs encoding these proteins can be cloned as described below and fused to the various IgG domains described in Example 1. The recombinant fusion proteins can be expressed and purified as described in Example 2 and 9. The purified fusion proteins can be tested in appropriate *in vitro* bioassays to determine their specific activities. DNA sequences, amino acid sequences and appropriate *in vitro* and *in vivo* bioassays for these proteins are well known in the art and are described in Aggarwal and Gutterman (1992; 1996), Aggarwal (1998), and Silvennoimem and Ihle (1996). Bioassays for these proteins also are provided in catalogues of commercial suppliers of these proteins such as R&D Systems, Inc, Endogen, Inc., and Gibco BRL.

1.**Cloning human alpha interferon.** Alpha interferon is produced by leukocytes and has antiviral, anti-tumor and immunomodulatory effects. There are at least 20 distinct alpha interferon genes that encode proteins that share 70% or greater amino acid identity. Amino acid sequences of the known alpha interferon species is given in Blatt et al., 1996). A "consensus" interferon that incorporates the most common amino acids into a single polypeptide chain has been described (Blatt et al, 1996). A hybrid alpha interferon protein may be produced by splicing different parts of alpha interferon proteins into a single protein (Horisberger and Di Marco, 1995). The following example describes construction of an alpha 2 interferon IgG fusion protein. Similar procedures can be used to create IgG fusions of other alpha interferon proteins.

DNA encoding human alpha interferon (IFN-α2) was amplified by PCR from human genomic DNA (CLONTECH). PCR reactions were carried out with BB93 (5>CGCGAATTCGGATATGT AAATAGATACACAGTG>3SEQ.ID.NO.44) and BB94 (5>CGCAAGCTTAAAAGATTTA AATCGTGTCATGGT>3)(SEQ.ID.NO.45). BB93 anneals to genomic sequences ~300 bp upstream (i.e. 5' to) of the IFN-alpha2 coding sequence and contains an *Eco* RI site for cloning purposes. BB94 anneals to genomic sequences ~100 bp downstream (i.e. 3' to) of the IFN-alpha2 coding sequence and contains a *Hin*d III site for cloning purposes. The PCR reaction employed 1X PCR reaction buffer (Promega Corp., Madison WI), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each oligonucleotide primer, 0.33 μg of genomic DNA and 0.4 units of Taq polymerase (Promega) in a 33μl reaction. The reaction consisted of 96°C for 3 minutes followed by 35 cycles of: [95°C for 60 sec., 58°C for 75 sec., 72°C for 90 sec.] followed by chilling the sample to 6°C. Reactions were carried out in a "Robocycler" thermal cycler (Stratagene Inc. , San Diego, CA). The resulting ~ 1 kb PCR product was digested with *Eco* RI and *Hin*d III and cl ned into similarly digested, and alkaline phosphatased, pCDNA3.1(+) (Invitrogen, San Diego, CA). A clone having the correct DNA sequence for IFN-α2 (Henco et al, 1985) was identified and designated pBBT160.

In order to construct and express gene fusions of IFN-α2 with IgG coding sequences the IFN-α2 gene was modified at the 5' and 3' ends using PCR based mutagenesis. pBBT160 plasmid DNA was used as template for PCR with forward primer BB108 (5' CGCAAGCTTGCCACCATGGCCTT GACCTTT GCTTTA-3') and reverse primer BB109 (5'- CGCGGATCCTCCGGATTCCTTACTT

5   CTTAAACTTTC-3'). Primer BB108 anneals to the 5' end of the coding sequence for the IFN-α2 secretion signal and the reverse primer, BB109, anneals to the 3' end of the IFN-α2 coding sequence. The resulting PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Henco et al., 1985) was designated

10   pCDNA3.1(+)::IFNAfus or pBBT190.

2. **Cloning human beta interferon.** Beta interferon is produced by fibroblasts and exhibits antiviral, antitumor and immunomodulatory effects. Beta interferon is the product of a single gene. DNA

15   encoding human beta interferon (IFN-β) was amplified by PCR from human genomic DNA (CLONTECH). PCR reactions were carried out with forward primer BB110 (5'-CGCAAGCTTGCCACCATGACCAACAAGTGTCTCCTC-3')(SEQ.ID.NO.48) and reverse primer BB111 (5'- CGCGGATCCTCCGGAGTTTCGGAGGTAACCTGTAAG-3')(SEQ.ID.NO.49). Primer BB110 anneals to the 5' end of the coding sequence for the IFN-β secretion signal and the reverse

20   primer, BB111, anneals to the 3' end of the IFN-β coding sequence. The resulting PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Derynck et al., 1980) was designated pCDNA3.1(+)::IFNBfus or pBBT191.

25   3. **Cloning human gamma interferon.** Gamma interferon is produced by activated T cells and exhbits anti-viral, antitumor and immunomodulatory effects. A cDNA encoding human gamma interferon (IFN-γ) was amplified by PCR from total RNA isolated from the human Jurkat T cell line (available from the American Type Culture Collection, Rockville, MD). The cells were grown in RPMI media supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. The cells were

30   activated *in vitro* for 6 hours with 1 μg/ml PHA-L (Sigma chemical Company, catalogue L-4144) and 50 ng/ml PMA (phorbol 12-myristate 13-acetate, Sigma Chemical Company, catalogue # P-1585) to induce IFN-γ expression prior to RNA isolation (Weiss et al., 1984; Wiskocil et al., 1985). RNA was isolated from the cells using an RNeasy Mini RNA isolation kit purchased from Qiagen, Inc. (Santa Clarita, CA) following the manufacturer's directions. Approximately 104 μg of total RNA was

35   isolated from 2.4×10^7 cells. First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp (Indianapolis, IN) and random hexamers were used as the primer. Subsequent PCR reacti ns using the products of the first strand synthesis as template were carried out with forward primer BB112 (5'-

CGCAAGCTTGCCACCATGAAATATACAAGTTATATC-3')(SEQ.ID.NO.50) and reverse primer

BB113 (5'- CGCGGATCCTCCGGACTGGGATGCTCTTCGACCTTG-3')(SEQ.ID.NO.51). Primer

BB112 anneals to the 5' end of the coding sequence for the IFN-γ secretion signal and the reverse

primer, BB113, anneals to the 3' end of the IFN-γ coding sequence. The resulting PCR product was

5 digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been

digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the

correct DNA sequence (Gray et al., 1982) was designated pCDNA3.1(+)::IFNGfus or pBBT192.

4. Construction of Interferon-IgG fusions. The IgG1-Fc coding sequence was fused to the

10 carboxyterminus of IFN-α2, IFN-β, and IFN-γ. The ~790 bp *Bam* HI – *Xba* I fragment was excised

from plasmid pBBT167 [described above in Example 1] and cloned into pBBT190, pBBT191 and

pBBT192 which had been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase.

Similarly, IgG4-Fc coding sequence also was fused to IFN-α2, IFN-β, and IFN-γ. The ~790 bp *Bam*

HI – *Xba* I fragment of plasmid pBBT158 [described above in Example 1] was excised, gel –purified

15 and cloned into pBBT190, pBBT191 and pBBT192 which had been digested with *Bam* HI and *Xba* I,

and treated with alkaline phosphatase. The IgG1-C_H coding sequence was fused to the

carboxyterminus of IFN-α2, IFN-β, and IFN-γ. The ~1080 bp *Bam* HI – *Xba* I fragment of plasmid

pBBT166 [described above in Example 1] was excised and cloned into pBBT190, pBBT191 and

pBBT192 which had been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. The

20 structures of the resulting recombinant plasmids were verified by restsriction endonuclease digestions

and agarose gel electrophoresis. These plasmids and the IFN-IgG fusion proteins that they encode are

listed in Table 8.

Table 8. Interferon – IgG Fusion Proteins

Expression Plasmid	IFN-Fusion Protein
pBBT193	IFN-α2-IgG1-Fc
pBBT194	IFN-α2-IgG4-Fc
pBBT220	IFN-α2-IgG1-C_H
pBBT195	IFN-β-IgG1-Fc
pBBT196	IFN-β-IgG4-Fc
pBBT221	IFN-β-IgG1-C_H
pBBT209	IFN-γ-IgG1-Fc
pBBT210	IFN-γ-IgG4-Fc
pBBT222	IFN-γ-IgG1-C_H

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5. Bioactivities f Interferon-IgG Fusion Proteins.

5.A. In Vitr Bi activities

In vitro biological assays for interferons include antiviral assays and cell proliferation inhibition assays. Proliferation of the human Daudi cell line (American Type Culture Collection, Rockville, MD) is inhibited by alpha, beta and gamma interferon and can be used to assay these proteins (Horoszewicz et al., 1979; Evinger and Pestka, 1981). Daudi cells are maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin. Bioassays are performed in this media using the procedures described above except that the number of cells added to each well of a 96 well plate can be 5-20 x 10^3 and the plates incubated at 37^0C for 3 to 4 days. The Daudi cells can be at early saturation density (1-2 x 10^6 cells/ml) before use in the assays for optimum effectiveness of the interferon. Serial dilutions of recombinant alpha, beta and gamma interferon (Endogen; R&D Systems; GibcoBRL, US Biological) can be analyzed in parallel. Recombinant alpha interferon has an IC_{50} (the concentration of protein required to inhibit proliferation by 50%) of approximately 5-30 pg/ml.

Bioactivities of alpha, beta and gamma interferons also can be measured using viral plaque inhibition assays. These assays measure the ability of the interferon protein to protect cells from viral infection. Methods for performing these assays are described in Ozes et al.,(1992) and Lewis (1987; 1995). Human HeLa or WISH cells (available from the American Type Cuture Collection) can be plated in 96-well plates (3x10^4 cells/well) and grown to near confluency at 37^0C. The cells can be washed and treated for 24 hour with serial 2-3-fold dilutions of each IFN-IgG fusion protein preparation. Vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) can then be added at a multiplicity of infection of 0.1 and the plates incubated for a further 24-48 hours at 37^0C. Additional controls can include samples without virus. When 90% or more of the cells have been killed in the virus-treated, no IFN control wells (determined by visual inspection of the wells), the cell monolayer can be stained with crystal violet (0.5% in 20% methanol) and absorbance of the wells read using a microplate reader. Alternatively, 20 µl of MTS/PMS mixture (CellTiter 96 AQueous One Solution, Promega Corporation, Madison, WI) can be added to the cell monolayers and absorbance the wells read at 490 nm after 1-4 hours later as described in Example 3. EC_{50} values (the amount of protein required to inhibit the cytopathic effect of the virus by 50%) can be used to compare the relative potencies of the fusion proteins and non-fused wild type proteins. Wild type IFN proteins protect cells from the cytopathic effects of VSV and EMCV and have specific activities of approximately 1x10^7 to 2x10^8 units/mg in this assay, depending upon the IFN species (Ozes et al., 1992).

The IFN-α-IgG and IFN-β-IgG fusion proteins listed in Table 8 were expressed in COS cells and purified as described in Examples 1 and 2. Non-reducing SDS-PAGE analysis showed that the IFN-α-IgG1-Fc and IFN-α-IgG1-C_H fusion proteins consisted predominantly of disulfide-linked dimers; however small am unts (<10%) of monomeric fusion protein was observed in all of the samples by non-reducing SDS-PAGE. The IFN-α-IgG1-C_H fusion protein also contained a significant amount of disulfide-linked aggregates, which failed to enter the gel. The IFN-α-IgG4-Fc fusion protein also was pred minantly dimeric; more monomer was present in this sample than in the IFN-α-

IgG1-Fc samples. The IFN-β-IgG1-Fc and IFN-β-IgG1-C$_H$ fusion proteins also were largely dimeric, with small amounts of monomeric fusion protein present in each sample. In contrast, the majority of the purified IFN-β-IgG4-Fc fusion protein was monomeric; the remainder was dimeric. Significant amounts of disulfide-linked aggregates were present in all of the purified IFN-β-IgG fusion proteins.

5   The purified IFN-α-IgG and IFN-β-IgG fusion proteins were assayed using the Daudi cell growth inhibition assay described above. All of the IFN-α-IgG and IFN-β-IgG fusion proteins were biologically active. IC$_{50}$ values for each protein were calculated and are shown in Table 9. Control recombinant IFN-α and IFN-β were purchased from Endogen, Inc. (Woburn, MA) and US Biological (Swampscott, MA), respectively.

10

Table 9. Bioactivities of IFN-α-IgG and IFN-β-IgG Fusion Proteins

Clone	Protein	IC$_{50}$ Range (ng/ml)[1]	Mean IC$_{50}$ (ng/ml)
-	rhIFN-α	0.015, 0.010	0.013
PBBT193	IFN-α2-IgG1-Fc	1.8, 2.5	2.1
PBBT194	IFN-α2-IgG4-Fc	2.5, 3.5	3.0
PBBT220	IFN-α2-IgG1-C$_H$	3.5	3.5
-	rhIFN-β	0.18, 0.3	0.24
PBBT195	IFN-β-IgG1-Fc	175, 200	188
PBBT196	IFN-β-IgG4-Fc	15, 15	15
PBBT221	IFN-β-IgG1-C$_H$	90	90

[1] Data from individual assays

15

IFN-gamma-IgG fusion proteins can be expressed in COS cells and purified by Protein A affinity chromatography as described in Example 2. Since IFN-gamma is sensitive to low pH, the fusion protein can be eluted from the affinity column using elution conditions that do not include low pH. One such method would be to elute the gamma interferon-IgG fusion protein with the Gentle Ag/Ab

20   elution buffers available from Pierce Chemical Company (catalogue # 21013 and 21014), followed by desalting of the protein on a desalting column.

5. B. In Vitro activity of IFN-α-IgKC

Plasmid DNA encoding the IFN-α-IgKC fusion protein described in Example 5 was

25   transfected into COS cells and c nditioned media collected after three days. Serial dilutions f the conditioned media were tested in the Daudi cell growth inhibition assay. Concentrations of the fusion protein were measured using an IFN-α2 ELISA kit purchased from Endogen, Inc. The conditioned media inhibited proliferati n of the Daudi cells. The IC$_{50}$ for the IFN-α-IgKC fusion was estimated to

44

be approximately 20 pg/ml (~ 0.69 pM). The control non-fused IFN-αprotein inhibited Daudi cell proliferation with an IC$_{50}$ of 6.5 pg/ml (~ 0.33 pM) in these experiments.

5. C. In Vivo Activity of IFN-IgG Fusion Proteins

5   IFN-α biological activity is relatively species-specific, which limits the range of preclinical animal models that can be studied. One model that can be used to measure *in vivo* efficacy of IFN-α-IgG fusion proteins is inhibition of human tumor xenograft growth in athymic nude mice. Human IFN-α2 is not active on mouse cells and inhibition of human tumor xenograft growth in nude mice occurs through a direct antiproliferative effect on the human tumor cells. IFN-α2 inhibits growth of a variety

10   of primary human tumor xenografts and human tumor cell lines in athymic mice (Balkwill et al., 1985; Balkwill, 1986; Johns et al., 1992; Lindner and Borden, 1997). The primary endpoint for the studies can be tumor volume in treated mice. Administration of the IFN-α-IgG fusion proteins will inhibit tumor growth (as measured by tumor volume) in the mice relative to vehicle-treated animals. Athymic nude mice can be purchased from a commercial vendor such as Charles River. Each mouse can be

15   injected with 2 x 10^6 NIH-OVCAR-3 or MCF-7 tumor cells (these cell lines are available from the American Type Culture collection) on day 0 and randomly assigned to test groups, consisting of ten mice each. An appropriate number of cells from another IFN-α-responsive tumor type can be substituted for the NIH-OVCAR-3 or MCF-7 cells. Tumor volumes can be determined at 4 day intervals by measuring the length and width of the tumors with calipers, as described by Linder and

20   Borden (1997). At time of sacrifice, the tumors can be excised and weighed. Mean tumor volumes +/- SEM for each test group can be calculated for each sampling point. Comparisons between groups can be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. Five μg of IFN-α2 administered once per day by subcutaneous injection inhibits growth of NIH-OVCAR-3 cells and MCF-7 cells in athymic mice by 80% after 6 weeks (Lindner and Borden,

25   1997). Either cell line can be used for these studies. The NIH-OVCAR-3 line (available from the ATCC) does not require estrogen for growth, as do the MCF-7 cells. Xenograft experiments with MCF-7 cells require that the mice be oophorectomized and implanted with estrogen pellets (Lindner and Borden, 1997). In initial experiments, different groups of mice can receive subcutaneous injections of 1 or 5 μg per injection of rIFN-α2, or 0.1 to 100 μg per injection IFN-α-IgG fusion proteins using

30   every day, every other day or every third day dosing schedules. Dosing can begin on day 2 following injection of the tumor cells into the mice. Control mice can receive vehicle solution only. In the every other day and every third day dosing experiments, an additional positive control group can receive daily subcutaneous injections of 5 μg rIFN-α2.

          In vivo efficacy of the IFN-β-IgG fusion proteins can be demonstrated in the same tumor

35   xenograft model using similar doses and dosing schedules.

6. IL-11-IgG fusi n proteins

6.A. Cloning IL-11. IL-11 stimulates development of megakaryocyte precursors of platelets. A cDNA encoding human IL-11 can be amplified by PCR from RNA isolated from human cell lines that express

IL-11 such as the human bladder carcinoma cell line 5637 and the HL60 and U937 leukemia cell lines (available from the American Type Culture Collection). PCR reactions can be carried out with forward primer IL-11F (5'- CGCAAGCTTGCCACCATGAACTGTGTTTGCCGCCTG -3')(SEQ.ID.NO.52) and reverse primer IL-11R (5'- CGCGGATCCTCCGGACAGCCGAGTCTTCAGCAGCAG –

5   3')(SEQ.ID.NO.53). Primer IL-11F anneals to the 5' end of the coding sequence for the IL-11 secretion signal and the reverse primer, IL-11R, anneals to the 3' end of the IL-11 coding sequence. The resulting PCR product can be digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones can be sequenced to identify one with the correct DNA sequence.

10   Alternatively, the IL-11coding sequence was amplified by PCR as two segments which were then subsequently spliced together to generate the full length clone by the technique of "overlap extension" as described above in Example 1, section C4 and by Horton et al. (1993). One segment, encoding amino acids 1 through 147, was amplified by PCR from single-stranded cDNA derived from total RNA extracted from the human bladder carcinoma cell line 5637 as detailed in Section 2 B.

15 above. A PCR reaction using the products of the first strand synthesis as template was carried out with forward primer BB265 (5> CGCAAGCTTGCCACCATGAACTG TGTTTGCCGCCTG >3)(SEQ.ID.NO.54) and reverse primer BB273 (5>GCGGGACATCAGGAG CTGCAGCCGGCGCAG >3)(SEQ.ID.NO.55). Primer BB265 anneals to the 5' end of the coding sequence for the IL-11 secretory signal sequence and the reverse primer, BB273, anneals to sequence

20 encoding amino acids 138 – 147 and spans the junction of exons 4 and 5 (McKinley et al., 1992). The ~450 bp product of this reaction was gel-purified and used in subsequent splicing reactions. The second segment, containing DNA sequences encoding encoding amino acids 142 through 197, was amplified by PCR from human genomic DNA (STRATAGENE). A PCR reaction using human genomic DNA as template was carried out with forward primer BB272 (5>CAGCTCCTGATGTCCCGCC TGGCCCTG

25 > 3)(SEQ.ID.NO.56) and reverse primer BB274 (5> AGTCTTCAGCAGCAGCAGTCCCC TCAC >3)(SEQ.ID.NO.57). BB272 anneals to sequence encoding amino acids 142 – 150 and spans the junction of exons 4 and 5. BB274 anneals to sequence encoding amino acids 189 – 197. The ~170 bp product of this reaction was gel-purified and used in subsequent splicing reactions.

        The gel purified ~450 bp and ~170 bp products were spliced together in a PCR reaction which

30 included the ~450 bp and ~170 bp products as template and forward primer BB265 (described above) and reverse primer BB275 (5> CGCGGATCCTCC GGACAGCCGAGTCTTCAGCAGCAG >3)(SEQ.ID.NO.58). BB275 anneals to the DNA sequence encoding amino acids 191-199. The ~620 bp product of this reaction was gel-purified, digested with *Hin* dIII and *Bam* HI and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated,

35 and gel purified. A clone with the correct DNA sequence was designated pCDNA3.1(+)::IL-11fus or pBBT298.

6.B. Construction f IL-11–IgG fusions. The IgG1-Fc coding sequence was fused to the carboxyterminus of IL-11. The ~790 bp *Bam* HI – *Xba* I fragment was excised from plasmid

pBBT174 [described above in Example 2, see Table 1] and cloned into pBBT298 which had been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. Similarly, IgG4-Fc coding sequence also was fused to the carboxyterminus of IL-11. The ~790 bp *Bam* HI – *Xba* I fragment of plasmid pBBT175 [described above in Example 2, see Table 1] was excised, gel–purified and cloned into pBBT298 which had been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. The IgG1-C_H and IgG4-C_H coding sequences also were fused to the carboxyterminus of IL-11. The IgG1-C_H and IgG4-C_H coding sequences were excised as ~1080 bp *Bam* HI – *Xba* I fragments from plasmids pBBT173 and pBBT184 [described above in Example 2, see Table 1] respectively, and cloned into pBBT298 which had been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. The structures of all the resulting recombinant plasmids were verified by restsriction endonuclease digestions and agarose gel electrophoresis. These plasmids and the IL-11-IgG fusion proteins they encode are listed in Table 10.

Table 10. IL-11 – IgG Fusion Proteins

Expression Plasmid	IL-11-Fusion Protein
pBBT336	IL-11-IgG1-Fc
pBBT337	IL-11-IgG4-Fc
pBBT338	IL-11-IgG1-C_H
pBBT339	IL-11-IgG4-C_H

An IL-11-IgG1-Fc direct fusion can be created by PCR using plasmid pBBT336 as the DNA template. One PCR reaction can use oligos IL11DFA (5' CTGAAGACTCGGCTGGA GCCCAAATCTTGTGACAAA-3')(SEQ.ID.NO.59) and BB82. The second PCR reaction can use oligos IL11DFB (5' ACAAGATTTGGGCTCCAGCCGAGTCTTCAGCAGCAG –3')(SEQ.ID.NO.60) and BB272. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB272. The approximate 800 bp PCR product can be gel-purified, digested with and *Sgr* AI III and *Sac* II, and cloned into similarly cut pBBT336 that had been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

An IL11-IgG4-Fc direct fusion can be created by PCR using plasmid pBBT337 as the DNA template. One PCR reaction can use oligos IL11DFC (5-CTGAAGACTCGGCTGGAGTC CAAATATGGTCCCCCA-3')(SEQ.ID.NO.61) and BB82. The second PCR reaction can use oligos IL11DFD (5'- ACCATATTTGGACTCCAGCCGAGTCTTCAGCAGCAG-3')(SEQ.ID.NO.62) and BB272. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB272. The approximate 800 bp PCR product can be gel-purified, digested with and *Sgr* AI and *Sac* II, and cloned into similarly cut pBBT337 that has been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

The B9-11 cell line (Lu et al., 1994), the T11 cell line (R & D Systems) or another IL-11-dependent cell line can be used t measure bioactivities f the IL-11-IgG fusion proteins. An IL-11-

dependent cell line can be created by culturing the B9 cell line (available from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in RPMI 1640 media containing 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin and 50-100 ng/ml of IL-11 for several days to several months, followed by limiting dilution cloning of the cells to select for individual lines.

6.C. In vivo efficacy of IL-11-IgG Fusion Proteins

The IL-11-IgG fusion proteins will stimulate increases in circulating platelets and megakaryopoiesis in normal mice or rats, similar to what is seen for hIL-11 and hTPO (Lok et al., 1994; Kaushansky et al., 1994; Neben et al., 1993; Yonemura et al., 1993). Efficacy of the IL-11-IgG fusion proteins can be tested in normal animals using every other day, every third day or single injection dosing schedules. Effectiveness of the IL-11-IgG fusion proteins also can be demonstrated in rodent chemotherapy-induced thrombocytopenia models (Hangoc et al., 1993; Leonard et al., 1994), using every other day, every third day or single injection dosing schedules.

Groups of mice or rats can receive subcutaneous injections of rhIL-11, IL-11-IgG fusion protein or placebo (vehicle solution) at specified intervals (daily, every other day, every third day) for up to seven days. A wide range of IL-11-IgG fusion protein doses can be tested in these initial experiments to increase the likelihood that one of the doses will be effective. The dose range of the IL-11-IgG fusion proteins to be tested can range from 0.002Xto 25X the optimum dose of IL-11 previously determined for these animal models. It is possible that administration of too much of the fusion proteins will impede megakarypoiesis due to toxicity. Control animals can receive vehicle solution only. Additional control groups can receive daily subcutaneous injections of rhIL-1. On day 7 the animals can be sacrificed and blood samples collected for complete blood cell count analysis. Hematopoietic tissues (liver and spleen) can be collected, weighed and fixed in formalin for histopathologic analyses to look for evidence of increased megakaryopoiesis. Bone marrow can be removed from various long bones and the sternum for unit particle preps and histopathologic analysis to look for evidence of increased megakaryopoiesis. Comparisons between groups can be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. $P < 0.05$ will be considered significant. The IL-11-IgG fusion proteins will stimulate increases in circulating platelets and megakaryopoiesis in the animals.

7. TPO-IgG fusion Proteins

7. A. Cloning TPO. Thrombopoietin (TPO) stimulates development of megakaryocyte precursors of platelets. A cDNA encoding human TPO was amplified by PCR from single-stranded cDNA prepared from human adult and fetal liver (CLONTECH, Inc.). Single-stranded cDNA prepared from fetal or adult kidney (CLONTECH) also can be used to amplify TPO. PCR reactions were carried out with forward primer BB343 (5'- CGCAAGCTTGCCACCATGGAGCTGACTGAATTGCTC – 3')(SEQ.ID.NO.63) and reverse primer BB344 (5'- CGCGGATCCTCCGGACCCTTCC

TGAGACAGATTCTG –3')(SEQ.ID.NO.64). Primer BB343 anneals to the 5' end of the coding

sequence for the TPO secretion signal and the reverse primer, BB344, anneals to the 3' end f the TPO

coding sequence.

5   The sequence encoding TPO contains an internal *Bam* HI site and this site was used to clone

the ~ 1000 bp TPO coding sequence as two segments; an amino-terminal *Hind* III - *Bam* HI fragment

of ~400 bp and a carboxy-terminal *Bam* HI fragment of ~600 bp. Each of these fragments was first

cloned and sequenced and subsequently ligated together through the internal *Bam* HI site to generate

the complete TPO coding sequence. The ~ 1,000 bp PCR product was digested with *Hin* dIII and *Bam*

HI to generate a *Hin* dIII - *Bam* HI fragment of ~400 bp and a *Bam* HI fragment of ~600. These

10   fragments were gel-purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III

and *Bam* HI, or only *Bam* HI, respectively, alkaline phosphatase treated, and gel purified. Clones with

the correct DNA sequences (Foster et al., 1994; Sohma et al., 1994) for each fragment were identified.

The 600 bp *Bam* HI fragment was then excised from the sequence-verified clone and cloned into *Bam*

HI-cut, alkaline phosphatase-treated and gel-purified pCDNA3.1(+) derivative containing the sequence

15   verified 400 bp *Hin* dIII - *Bam* HI fragment. The resulting clones were analyzed by restriction

endonuclease digestion and PCR to identify those that carried the 600 bp *Bam* HI fragment cloned in

the proper orientation i.e., so that the coding sequence for TPO is properly reconstituted. One properly

oriented clone was designated pCDNA3.1(+)::TPOfus, or pBBT355, and used in further constructions.

20   **7. B. Construction of TPO–IgG fusions.** Fusions of IgG coding sequences to the carboxy-terminus of

TPO were constructed using procedures analogous to those used to construct the IL-11-IgG fusions

with the exception that restriction endonuclease *Bsp* EI was used in place of *Bam* HI because the

coding sequence for TPO contains a *Bam* HI site. The sequence employed to encode the ser-gly-gly-

ser-gly-gly-ser (SEQ.ID.NO.3) linker peptide in all of the constructs described in Examples 1, 2 and 10

25   contains a single *Bsp* EI recognition site (TCCGGA) and this site does not occur within the

pCDNA3.1(+) vector or the IgG1-Fc, IgG4-Fc, IgG1-C$_H$, and IgG4-C$_H$ coding sequences.

The IgG1-Fc coding sequence was fused to the carboxyterminus of TPO. The ~790 bp *Bsp*

EI – *Xba* I fragment was excised from plasmid pBBT174 [described above in Example 2, see Table 1]

and cloned into pBBT355 which had been digested with *Bsp* EI and *Xba* I, and treated with alkaline

30   phosphatase. Similarly, IgG4-Fc coding sequence also was fused to the carboxyterminus of TPO. The

~790 bp *Bsp* EI – *Xba* I fragment of plasmid pBBT175 [described above in Example 2, see Table 1]

was excised, gel –purified and cloned into pBBT355 which had been digested with *Bsp* EI and *Xba* I,

and treated with alkaline phosphatase. The IgG1-C$_H$ and IgG4-C$_H$ coding sequences also were fused to

the carboxyterminus of TPO. The IgG1-C$_H$ and IgG4-C$_H$ coding sequences were excised as ~1080 bp

35   *Bsp* EI – *Xba* I fragments from plasmids pBBT173 and pBBT184 [described above in Example 2, see

Table 1] respectively, and cloned into pBBT355 which had been digested with *Bsp* EI and *Xba* I, and

treated with alkaline phosphatase. The structures of all the resulting recombinant plasmids were

verified by restsriction endonuclease digestions and agarose gel electrophoresis. Table 11 lists these

plasmids and the TPO-IgG fusion proteins they encode.

Table 11. TPO – IgG Fusion Proteins

Expression Plasmid	TPO Fusion Protein
pBBT340	TPO-IgG1-Fc
pBBT341	TPO-IgG4-Fc
pBBT342	TPO-IgG1-C_H
pBBT343	TPO-IgG4-C_H

The amino-terminal domain of TPO, consisting of amino acids 1 – 153 of the mature TPO

5   protein has been shown to be sufficient for megakaryopoiesis (Bartley et al., 1994, de Sauvage et al., 1994). This domain could be subcloned by PCR from the TPO coding sequence in pBBT355 (described above) and fused to immunoglobulin sequences. The PCR reaction would be carried out using forward primer BB343 (described above) and reverse primer TPO153rev (5> CGC GGA TCC TCC GGA CCT GAC GCA GAG GGT GGA CCC >3)(SEQ.ID.NO.65). Primer TPO153rev anneals

10   to the TPO sequence encoding amino acids 147 through 153 and adds a portion of the flexible linker containing a *Bam* HI site. The template for this reaction would be pBBT355 DNA. The resulting PCR product of ~ 600 bp would be digested with *Bam* HI and the resulting ~ 105 bp *Bam* HI fragment would be gel purified, cloned in pUC18 and sequenced. A clone having the correct sequence would then be used as a source of plasmid DNA from which the ~105 bp *Bam* HI fragment would be gel

15   purified. This fragment would be used to replace the ~600 bp *Bam* HI fragment present in pBBT340, pBBT341, pBBT342 and pBBT343 (which are all described above). This will result in construction of fusion proteins consisting of amino acids 1 – 153 of TPO fused via the ser-gly-gly-ser-gly-gly-ser linker to IgG1-Fc, IgG4-Fc, IgG1-C_H, and IgG4-C_H respectively.

A TPO-IgG1-Fc direct fusion can be created by PCR using plasmid pBBT340 as the DNA

20   template. One PCR reaction can use oligos TPODFA (5' CTGTCTCAGGAAGGGGAG CCCAAATCTTGTGACAAA-3')(SEQ.ID.NO.66) and BB82. The second PCR reaction can use oligos TPODFB (5' ACAAGATTTGGGCTCCCCTTCCTGAGACAGATTCTG –3)(SEQ.ID.NO.67) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The approximate 1700 bp PCR product cand be gel-purified,

25   digested with and *Hin*d III and *Sac* II, and cloned into similarly cut pBBT340 that had been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

A TPO-IgG4-Fc direct fusion can be created by PCR using plasmid pBBT341 as the DNA template. One PCR reaction can use oligos TPODFC (5-CTGTCTCAGGAAGGGGAGTCCAAA TATGGTCCCCCA-3')(SEQ.ID.NO.68) and BB82. The second PCR reaction can use oligos TPODFD

30   (5'- ACCATATTTGGACTCCCCTTCCTGAGACAGATTCTG-3'SEQ.ID.NO.69) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The approximate 2000 bp PCR product can be gel-purified, digested with and *Hin*d III and *Sac* II, and cloned into similarly cut pBBT341 that has been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

Similar methods could be used to construct analogous direct fusi ns encoding only amino acids 1-153 of TPO fused to IgG-Fc domains.

The cell line M-O7e (Avanzi et al., 1988; available from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) can be used to measure TPO-IgG fusion protein bioactivity.

7.C. In vivo efficacy of TPO-IgG Fusion Proteins

The TPO-IgG fusion proteins will stimulate increases in circulating platelets and megakaryopoiesis in normal mice or rats, similar to what is seen for hIL-11 and hTPO (Lok et al., 1994; Kaushansky et al., 1994; Neben et al., 1993; Yonemura et al., 1993; Kaszubska et al., 2000). Efficacy of the TPO-IgG fusion proteins can be tested in normal animals using every other day, every third day or single injection dosing schedules. Effectiveness of the TPO-IgG fusion proteins also can be demonstrated in rodent chemotherapy-induced thrombocytopenia models (Hanggoc et al., 1993; Leonard et al., 1994), using every other day, every third day or single injection dosing schedules.

Groups of mice or rats can receive subcutaneous injections of rhTPO, TPO-IgG fusion protein or placebo (vehicle solution) at specified intervals (daily, every other day, every third day) for up to seven days. A wide range of TPO-IgG fusion protein doses can be used in these initial experiments to increase the likelihood that one of the doses will be effective. The dose range of the TPO-IgG fusion proteins to be tested can range from 0.002X to 25X the optimum dose of TPO previously determined for these animal models. It is possible that administration of too much of the fusion proteins will impede megakarypoiesis due to toxicity. Control animals can receive vehicle solution only. Additional control groups can receive daily subcutaneous injections of rhTPO. On day 7 the animals can be sacrificed and blood samples collected for complete blood cell count analysis. Hematopoietic tissues (liver and spleen) can be collected, weighed and fixed in formalin for histopathologic analyses to look for evidence of increased megakaryopoiesis. Bone marrow can be removed from various long bones and the sternum for unit particle preps and histopathologic analysis to look for evidence of increased megakaryopoiesis. Comparisons between groups can be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. $P < 0.05$ will be considered significant. The TPO-IgG fusion proteins will stimulate increases in circulating platelets and megakaryopoiesis in the animals.

8. GM-CSF-IgG Fusion Proteins

8.A. Cloning GM-CSF:
A cDNA encoding human Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) was amplified by PCR from total RNA prepared from the human bladder carcinoma cell line 5637 as detailed in Section 2 B. above. A PCR reaction using the products of the first strand synthesis as template was carried out with forward primer BB263 (5> CGCAAGCTTGCCACCATGTGGCTGCAGAGC CTGCTG >3)(SEQ.ID.NO.70) and reverse primer BB264 (5>CGCGGATCCTCCGGACTCCTGGACTGGCT CCCAGCA >3)(SEQ.ID.NO.71). Primer BB263 anneals to the 5' end of the coding sequence for the GM-CSF secretion signal and the reverse

primer, BB264, anneals to the 3' end of the GM-CSF coding sequence. The resulting ~ 450 bp PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Cantrell et al., 1985) was designated pCDNA3.1(+)::GM-CSFfus or

5   pBBT267.

Alternatively, a cDNA encoding human GM-CSF can be amplified by PCR from RNA isolated from a cell line that expresses GM-CSF such as the human T cell line HUT 102 (available from American Type Culture Collection) or from human peripheral blood lymphocytes or the human Jurkat T cell line that had been activated with 20 μg/ml concanavalin A (Sigma Chemical Company)

10   and 40 ng/ml phorbol myristate acetate (PMA, Sigma Chemical Company). PCR reactions can be carried out with forward primer BB263and reverse primer BB264. The resulting ~ 450 bp PCR product can be digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones can be sequenced to identify one with the correct DNA sequence.

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8. B. Construction of GM-CSF–IgG (GF-IgG) fusions. The IgG1-Fc coding sequence can be fused to the carboxy-terminus of GM-CSF. The ~790 bp *Bam* HI – *Xba* I fragment excised from plasmid pBBT174 [described above in Example 2, see Table 1] can be cloned into pBBT267 that has been

20   digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. Similarly, IgG4-Fc coding sequence also can be fused to the carboxy-terminus of GM-CSF. The ~790 bp *Bam* HI – *Xba* I fragment excised from plasmid pBBT175 [described above in Example 2, see Table 1] can be cloned into pBBT267 that has been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. The IgG1-C_H and IgG4-C_H coding sequences also can be fused to the carboxy-terminus of GM-CSF.

25   The IgG1-C_H and IgG4-C_H coding sequences were excised as ~1080 bp *Bam* HI – *Xba* I fragments from plasmids pBBT173 and pBBT184 [described above in Example 2, see Table 1] respectively, and can be cloned into pBBT267 that has been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. The structures of all the resulting recombinant plasmids can be verified by restriction endonuclease digestions and agarose gel electrophoresis. Bioactivity of the GM-CSF-IgG fusion

30   proteins can be measured using the TF-1 cell line (available from the American Type Culture Collection).

A GM-CSF-IgG1-Fc direct fusion can be created by PCR using plasmid pcDNA3.1::GM-CSF-IgG1-Fc as the DNA template. One PCR reaction can use oligos GMCSFDFA (5' GAGCCAGTCCAGGAG GAGCCCAAATCTTGTGACAAA-3') and BB82. The second PCR reaction can use oligos

35   GMCSFDFB (5' ACAAGATTTGGGCTCCTCCTGGACTGGCTCCCAGCA –3) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The approximate 1150 bp PCR product can be gel-purified, digested with and *Hind* III and *Sac* II, the resulting ~ 675 bp *Hind* III/*Sac* II fragment gel-purified and cloned

into similarly cut plasmid pcDNA3.1::GM-CSF-IgG1-Fc that had been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

A GM-CSF-IgG4-Fc direct fusion can be created by PCR using plasmid pcDNA3.1::GM-CSF-IgG4-Fc as the DNA template. One PCR reaction can use oligos GMCSFDFC

5   (5GAGCCAGTCCAGGAGGAGTCCAAATATGGTCCCCCA-3')(SEQ.ID.NO.76) and BB82. The second PCR reaction can use oligos GMCSFDFD (5'- ACCATATTTGGACTCCTCCT GGACTGGCTCCCAGCA-3')(SEQ.ID.NO.77) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The approximate 1150 bp PCR product can be gel-purified, digested with and *Hin*d III and *Sac* II, the ~675

10   bp *Hin*d III/*Sac* II fragment gel-purified and cloned into similarly cut plasmid pcDNA3.1::GM-CSF-IgG4-Fc that has been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

Bioactivity of the GM-CSF-IgG fusion proteins can be measured using the TF-1 cell line (available from the American Type Culture Collection).

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8. C. In Vivo efficacy of GM-CSF-IgG Fusion Proteins

GM-CSF stimulates granulopoiesis and an increase in circulating neutrophil levels in normal and neutropenic humans and animals. Efficacy of the human GM-CSF-IgG fusion proteins can be determined in non-human primates (Donahue et al., 1986a, 1986b ; Mayer et al., 1987a, 1987b) and

20   dogs (Schuening et al., 1989; Mayer et al., 1990), where injection of the protein can stimulate an increase in circulating neutrophil levels over time. Alternatively, analogous IgG fusion proteins to those described here can be made using mouse GM-CSF (Gough et al., 1984; 1985; Kajigaya et al., 1986; Cantrell et al., 1985) and the proteins expressed and purified as described for the human GM-CSF cysteine muteins. In vitro bioactivities of the proteins can be measured using the murine NFS60

25   or DA-3 cells lines. Efficacy of the mouse GM-CSF-IgG fusion proteins can be measured in mice made neutropenic by injection of cyclophosphamide at a dose of 200 mg/kg (Mayer et al., 1991; Gamba-Vitalo et al., 1991) using once per day, every other day or every third day dosing schedules for the murine GM-CSF-IgG fusion proteins. Doses of the GM-CSF –IgG fusion proteins ranging from 0.5 μg/kg to 650 μg/kg per injection can be tested.

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9. Stem Cell Factor-IgG Fusion Proteins

9. A. Cloning Stem Cell Factor (SCF): SCF regulates development of hematopoietic progenitor cells. A cDNA encoding human SCF can be amplified by RT-PCR using RNA isolated from HepG2, 5637 or HT-1080 cell lines (Martin et al., 1990; the HepG2 and 5637 cell lines are available from the ATCC,

35   Rockville, MD). PCR reactions can be carried out with forward primer SCF-F (5'- CGCAAGCTTGCCACC<u>ATGAAGAAG</u> <u>ACACAAACT</u> –3')(SEQ.ID.NO.78) and reverse primer SCF-R (5'-CGCGGATCCTCCGGA <u>GTGTAGGCTGGAGTCTCCAGG</u> –3')(SEQ.ID.NO.79). SCF DNA sequences in the primers are underlined. Primer SCF-F anneals to the 5' end of the coding sequence for the SCF secretion signal

and the reverse primer, SCF-R, anneals to the 3' end of the SCF coding sequence, beginning at the junction of the extracellular and transmembrane domains. Other reverse PCR primers can be used to create truncated forms of the SCF extracellular domain, in particular a form that terminates following Ala-174 of the mature protein, by substituting appropriate nucleotides for the SCF DNA sequence listed in SCF-R. The resulting PCR product can be digested with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones can be sequenced to identify one with the correct DNA sequence. IgG1-Fc, IgG4-Fc, IgG1-C_H, IgG4-C_H and kappa light chain constant regions can be fused to the carboxy-terminus of the extracellular domain of SCF as described in Examples 1 and 5.

10   The cell line TF-1 (Kitamura, 1989; available from the American Type Culture Collection, Rockville, MD) can be used to measure bioactivity of SCF-IgG fusion proteins.

Direct fusions of the extracellular domain of SCF to various IgG domains can be constructed using procedures similar to those described in Example 4 for constructing EPO-IgG and G-CSF-IgG direct fusions. A SCF-IgG1-Fc direct fusion can be created by PCR using plasmid pcDNA3.1::SCF-

15   IgG1-Fc7AA as the DNA template. One PCR reaction can use oligos SCFDFF (5'-GACTCCAGCCTACACGAGCCCAAATCTTGTGACAAA-3')(SEQ.ID.NO.80) and BB82. The second PCR reaction used oligos SCFDFR (5'-ACAAGATTTGGGCTCGTGT AGGCTGGAGTCTCCAGG-3'SEQ.ID.NO.81) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The

20   PCR product can be gel-purified, digested with and *Hin*d III and *Sac* II, and cloned into similarly cut pcDNA3.1::SCF-IgG1-Fc7AA that had been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

A SCF-IgG4-Fc direct fusion can be created by PCR using plasmid pcDNA3.1::SCF-IgG4-Fc7AA as the DNA template. One PCR reaction can use oligos SCFDFC (5'-

25   GACTCCAGCCTACACGAGTCCAAATATGGTCCCCCA-3')(SEQ.ID.NO.82) and BB82. The second PCR reaction can use oligos SCFDFD (5'- ACCATATTTGGACTCGTGTA GGCTGGAGTCTCCAGG-3') (SEQ.ID.NO.83) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The PCR product can be gel-purified, digested with and *Hin*d III and *Sac* II, and cloned into similarly cut

30   pcDNA3.1::SCF-IgG4-Fc7AA that has been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

10. **Flt-3L-IgG fusion Proteins:** Flt-3L (Lyman et al., 1993; Hannum et al., 1994) is a membrane bound cytokine that regulates development of hematopoietic stem cells. A cDNA encoding human Flt-

35   3L can be amplified by PCR from single-stranded cDNA prepared from adult or fetal liver, kidney, heart, lung, or skeletal muscle, which is available from commercial sources such as CLONTECH and Stratagene, Inc.. PCR reactions can be carried ut with forward primer fltF (5'-CGCAAGCTTGCCACCATGACAGTGCTGGCGCCAGCC -3') (SEQ.ID.NO.84) and reverse primer fltR (5'- CGCGGATCCTCCGGAAGGGGGCTGCGGGGCTGTCGG -3')(SEQ.ID.NO.85). Flt-3L

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DNA sequences in the primers are underlined. Primer fltF anneals to the 5' end of the coding sequence for the Flt-3L secretion signal and the reverse primer, fltR, anneals to the 3' end of the Flt3 coding sequence beginning at the junction of the extracellular and transmembrane domains. Other reverse PCR primers can be used to create truncated forms of the Flt-3L extracellular domain by substituting

5   appropriate nucleotides for the Flt-3L DNA sequence listed in primer fltR. The resulting PCR product can be digested with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones can be sequenced to identify one with the correct DNA sequence. IgG1-Fc, IgG4-Fc, IgG1-C$_H$, IgG4-C$_H$ and kappa light chain constant regions can be fused to the carboxy-terminus of the extracellular

10   domain of Flt-3L as described in Examples 1 and 5. Ba/F3 cells transfected with the human Flt-3 receptor (Lyman et al.,1993; Hannum et al., 1994) can be can be used to measure bioactivity of Flt-3L-IgG fusion proteins. Ba/F3 cells are available from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Direct fusions of the extracellular domain of Flt-3L to various IgG domains can be constructed

15   using procedures similar to those described in Example 4 for constructing EPO-IgG and G-CSF-IgG direct fusions. A Flt-3L-IgG1-Fc direct fusion can be created by PCR using the pcDNA3.1::Flt-3L-IgG1 plasmid described above as the DNA template. One PCR reaction can use oligos FltDFA (5'-GCCCCGCAGCCCCTGAGCCCAAA
TCTTGTGACAAA-3' (SEQ.ID.NO.86) and BB82. The second PCR reaction can use oligos Flt3DFB

20   (5'-ACAAGATTTGGGCTCAGGGGGCTGCGGGGCTGTCGG-3')(SEQ.ID.NO.87) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The PCR product can be gel-purified, digested with and *Hin*d III and *Sac* II, and cloned into similarly cut pcDNA3.1::SCF-IgG1-Fc7AA that had been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

25   A Flt-3L-IgG4-Fc direct fusion can be created by PCR using plasmid pcDNA3.1::Flt3-IgG4-Fc7AA as the DNA template. One PCR reaction can use oligos FltDFC (5'-GCCCCGCAGCCCCCT
GAGTCCAAATATGGTCCCCCA-3')(SEQ.ID.NO.88) and BB82. The second PCR reaction can use oligos FltDFD (5'- ACCATATTTGGACTCAGGGGGCTGCGGGGCTGTCGG-3')(SEQ.ID.NO.89) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third

30   PCR reaction using oligos BB82 and BB91. The PCR product can be gel-purified, digested with *Hin*d III and *Sac* II, and cloned into similarly cut pcDNA3.1::SCF-IgG4-Fc7AA that has been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.

Example 11
Multimeric Cytokine and Growth Factor Fusion Proteins

35   Bioactive fusion proteins also can be created by constructing multimeric fusion proteins of the growth factors and cytokines mentioned in this application. These multimeric fusion proteins can be constructed as described for the IgG fusion proteins except that a second growth factor/cytokine protein can be substituted for the IgG domain. The two growth factors/cytokines can be joined together with or

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without linker amino acids between the two growth factors/cytokines. Suitable peptide linkers include those described in Examples 1 and 4. The fusion proteins can be homodimeric, heterodimeric, homomultimeric (comprising three or more copies of the same growth factor/cytokine) or heteromultimeric (comprising two or more different growth factors/cytokines). The most carboxy-
5   terminal cytokine/growth factor domain can be modified using procedures such as PCR to delete the protein's natural signal sequence and add, if desired, a short peptide linker sequence preceding the first amino acid of the mature protein sequence. The linker sequence could include a restriction enzyme site to facilitate joining to the amino-terminal cytokine/growth factor domain. In multimeric fusion proteins the cytokine/growth factor domains not at the amino- or carboxy-terminus of the protein can
10   be modified to delete the natural signal sequence and termination codon and add, if desired, peptide linkers to the amino- and carboxy-termini of the protein. The fusion proteins can expressed in COS cells following transfection, purified and tested in appropriate *in vitro* bioassays. The fusion proteins also can be expressed in stably transformed eukaryotic cells such as mammalian cells, as described in Example 12.

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Example 12
Expression of IgG Fusion Proteins and Multimeric Growth Factors/Cytokines in Stably Transformed Eukaryotic Cells

20   The IgG Fusion proteins and mutimeric cytokines/growth factors described in the preceding Examples also can be expressed in stably transformed eukaryotic cells such as insect cells or mammalian cells using well established procedures. The IgG fusion proteins and multimeric cytokines/growth factors are then purified from the conditioned media. Stably transfected CHO cell lines are widely used for high-level expression of recombinant proteins (Geisse et al. 1996; Trill et al.
25   1995). In CHO cells, high level expression of chromosomally integrated heterologous genes can be achieved by gene amplification. Typically the gene of interest is linked to a marker gene for which amplification is selectable. A variety of genes which provide selections for amplification have been described (Kaufman 1990) but murine dihydrofolate reductase (dhfr) is frequently employed. Amplification of this gene confers resistance to the folate analog methotrexate (MTX) and the level of
30   resistance increases with dhfr gene copy number (Alt et al., 1978). Utility of MTX selection is enhanced by the availability of CHO cell lines that are deficient in dhfr (Urlaub and Chasin, 1980).

        One skilled in the art can construct expression vectors for the IgG fusion proteins or multimeric cytokines/growth factors that incorporate the murine dhfr gene into the commercially available pCDNA3.1 expression vector (Invitrogen), which includes the neomycin phosphotransferase
35   (NPT) gene that confers resistance to G418. The murine dhfr expression vector pdhfr2.9 is available from the American Type Culture Collection (catalogue No.37165). This dhfr gene is selectable in dhfr⁻ CHO cell lines and can be amplified by standard selections for MTX resistance (Crouse et al 1983). The dhfr coding sequence can be excised fr m pdhfr2.9 as a ~ 900 bp *Bgl* II fragment and cloned into the unique *Bam* HI site of the polylinker f the expression vector pREP4 (Invitrogen). This construct

will position the gene downstream of the strong RSV promoter, which is known to function in CHO cells (Trill et al, 1995) and upstream of a polyadenylation site derived from SV40. This dhfr expression cassette can then be excised from pREP4 as a *Sal* I fragment since *Sal* I sites closely flank the promoter and polyA addition site. Using oligonucleotide linkers this *Sal* I fragment can be cloned

5   into the unique *Bgl* II site of pCDNA3.1, creating pCDNA3.1(+)::dhfr$^+$. DNA encoding the IgG fusion protein or mutimeric cytokine/growth factor can subsequently be cloned into the polylinker region of this pCDNA3.1(+) derivative under the control of the CMV promoter. For example, the EPO-IgG1-Fc or G-CSF-IgG1-Fc genes could be excised from plasmids pBBT180 and pBBT174, respectively as *Hind* III – *Xba* I fragments and cloned into *Hind* III-*Xba* I-cut pCDNA3.1(+)::dhfr$^+$. Other unique

10   restriction enzyme sites can be used if these restriction enzyme sites are present in the genes of interest. Alternatively, co-transfection of pCDNA3.1(+)::dhfr$^+$ and pCDNA3.1 derivatives expressing the IgG fusion proteins or mutimeric cytokines/growth factors could be employed to obtain dhfr$^+$ CHO cell lines expressing the protein of interest (Kaufmann, 1990).

          Endotoxin free plasmid DNAs are preferred for transfecting mammalian cells such as COS or

15   dhfr$^-$ CHO cells. Dhfr$^-$ CHO cell lines can be obtained from a number of sources such as Dr. L. Chasin at Columbia University (CHO K1 DUKX B11) or from the American Type Culture Collection (CHO duk$^-$, ATCC No. CRL-9096). The cells can be cultured in F12/DMEM medium supplemented with 10% FBS, glutamine, glycine, hypoxanthine, and thymidine (Lucas et al., 1996). Transfections can be carried out by electroporation or by using transfection reagents well known to those of skill in the art

20   such as LipofectAMINE (Gibco BRL), using the vendor protocols and / or those described in the literature (Kaufman, 1990). One can select for dhfr$^+$ transfectants in F12/DMEM supplemented with 7% dialyzed FCS and lacking glutamine, glycine, hypoxanthine, and thymidine (Lucas et al., 1996). Alternatively one can select for G418 resistance (encoded by the NPT gene of pCDNA3.1) and subsequently screen transfectants for the dhfr$^+$ phenotype. Dhfr$^+$ clones can be expanded in selection

25   medium and culture supernatants screened for expression of the IgG fusion proteins or multimeric cytokines/growth factors using commercially available ELISA kits specific for the growth factor or cytokine (available from R & D Systems, Endogen, Inc. and Diagnostic Systems Laboratories) or by Western blot using appropriate antisera (available from R&D Systems, Endogen, Inc. and Diagnostic Systems Laboratories). Clones expressing the IgG fusion proteins or mutimeric cytokines/growth

30   factors can then be pooled and subjected to multiple rounds of selection for MTX resistance at increasing drug concentration as described by Kaufman (1990). After each round of MTX selection, individual clones can be tested for expression of the IgG fusion proteins or mutimeric cytokines/growth factors. These procedures are well described in the literature and have been used to express a variety of heterologous protein in CHO cells (reviewed in Kaufman, 1990).

35   The IgG fusion proteins can be purified from the conditioned medium of CHO cells. After removal f the CHO cells by centrifugation, the IgG fusion proteins can be purified from the supernatant by Protein A affinity column chromatography using protoc ls similar to those described in Example 2, followed by additional chr matgraphy steps as needed. Dimeric IgG-Fc and IgG-C$_H$ fusions can be purified further by size-exclusion chromatography to remove monomers and aggregates.

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Additional column chromatography steps could include Blue Sepharose, hydroxyapatite, reversed phase, hydrophobic interaction, and ion-exchange chromatography. These techniques are w ll known to those of skill in the art.

Mutimeric cytokines/growth factors and cytokine/growth factor-light chain constant region fusions also can be purified from the conditioned medium of CHO cells. After removal of the CHO cells by centrifugation, the mutimeric cytokines/growth factors and cytokine/growth factor-light chain constant region fusions can be purified from the supernatant by column chromatography using techniques well known to those of skill in the art. These column chromatography steps could include Blue Sepharose, hydroxyapatite, reversed phase, hydrophobic interaction, size exclusion and ion-exchange chromatography. The proteins also can be purified by affinity chromatography using monoclonal antibodies specific for the cytokine/growth factor or light chain constant region.

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While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention.